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Intestinal responses to *Clostridium perfringens* in broilers

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Declaration

I declare that I have completed the present thesis. This is my own work and any assistance has been duly acknowledged. The work has not been submitted for any other degree or professional qualification.

Katherine M Russell

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Abstract

Clostridium perfringens is the aetiological agent of Necrotic enteritis (NE); a disease that impacts on the health and welfare of broilers. This disease is a large cost to the industry and presents as lesions in the small intestine hindering productivity. Antibiotics are commonly used to treat NE but as pressure increases to limit their use further information about disease onset and broiler responses to the bacteria and it's virulence factors during infection is required to implement new preventative measures and treatments.

NetB is a secreted toxin from *C. perfringens* which has an important role in NE onset. Using an *in situ* intestinal loop model we have been able to characterise: I) temporal broiler responses to NetB positive bacterial culture supernatant (Chapter 2), ii) early host responses to different isolates possessing NetB (virulent) or not (avirulent) in the presence or absence of bacterial cells (Chapter 3) and iii) the responses of two commercial broiler breeds (Chapter 4) four hours post exposure. Samples collected from these experiments have been used for histology, mRNA expression and immunohistology.

We have shown differences in mRNA expression in the duodenum of broilers after exposure to *C. perfringens* cells as well as the culture supernatant from the isolates used after four hours. The presence of bacteria cells resulted in up-regulation of pro-inflammatory cytokine, IFN-γ, mRNA, whereas it resulted in down-regulation of B-LA, mRNA a gene involved in presentation of pathogens to immune cells. IL-6 mRNA expression was also reduced in the

presence of virulent isolates. This could indicate a possible evasion strategy for *C. perfringens* in broilers. Immunohistochemical analysis indicated that slower growing broilers have increased numbers of immune cells (macrophages and γδ T cells) in their duodenum compared with faster growing broilers, although this did not appear to have an effect on mRNA expression levels of pro-inflammatory cytokines, 4h post antigen infusion. Overall we detect greater changes when bacteria are included with culture supernatant and have highlighted possible mechanisms for *C. perfringens* to avoid the broiler immune system.

Induction of NE in the literature requires pre-disposing factors, including co-infection with other intestinal pathogens and dietary manipulation of the host. The final experiment trialled protocols administering a virulent isolate of *C. perfringens* in-feed and via gavage along with an increased protein source to induce NE (Chapter 5). These models were not considered to be consistent for further investigation of NE in the future.

Lay Summary

Clostridium perfringens causes Necrotic enteritis (NE); a bacterial disease that impacts on the health and welfare of chickens produced for their meat (broilers). NE produces lesions in the small intestine preventing absorption of nutrients, efficient growth of broilers and therefore is large cost to the industry. Antibiotics are commonly used to treat NE but as pressure increases to limit their use further information about disease onset and broiler responses to the bacteria is required to implement new preventative measures and treatments.

NetB is a secreted toxin from *C. perfringens* which has an important role in NE onset. Using a surgical model we have been able to compare the responses of broilers when they are administered: i) the NetB toxin or a control preparation (Chapter 2) and ii) toxin with or without the bacterial cells (Chapter 3). We have also used different commercial breeds (Chapter 4). Samples from these experiments have been used to evaluate the intestinal structure, the types of cells present to fight infection and to determine what responses are induced in these cells.

We have shown differences in the responses detected in the small intestine after exposure to *C. perfringens* bacteria cells as well as the toxins. These differences were not always the same, indicating that the presence of bacteria results in different responses compared to that of the toxin.

Bacteria resulted in increase in markers of local inflammation whereas they reduced the levels of a marker associated with the initiation of the immune response indicating this bacterium may attempt to avoid detection in

broilers. One commercial breed had significantly more immune cells in the small intestine than the other.

We also tested a number of different protocols to reproduce the disease in chickens, such as administering *C. perfringens* and the NetB toxin in-feed and via gavage along with an increased protein source (Chapter 5). However, none of these models consistently resulted in lesions of NE, and thus will not be used in the future.

We have highlighted that *C .perfringens* bacteria induce different responses in comparison to the toxins they produce and they may try to avoid detection in the broiler intestine. Further investigation is required to determine whether this could contribute to the onset of NE in broilers and whether it could be targeted to treat this disease.

Publications

Research articles (peer reviewed)

S. Athanasiadou, **KM Russell**, P Kaiser, T Kanellos, STG Burgess, MA Mitchell, RE Clutton, SW Naylor, MR Hutchings, NH Sparks. Genome-wide transcriptomic analysis identifies pathways affected by the infusion of *Clostridium perfringens* culture supernatant in the duodenum of broilers in situ. The Journal of Animal Science. 93:6,p3152-3163

Conference abstracts (peer reviewed)

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KM Russell, P Kaiser, NH Sparks, V Parreira, JF Prescott, MA Mitchell, RE Clutton, T Kanellos, S Athanasiadou (2014) Host responses to virulent and avirulent strains of *Clostridium perfringens* as measured *in situ* in broilers. BSAS and AVTRW, Science into Practice - planning for intensification, Nottingham, UK. Abstract 161. Oral presentation

KM Russell, P Kaiser, NH Sparks, V Parreira, JF Prescott, T Kanellos, S Athanasiadou (2014)Local responses to virulent and avirulent *Clostridium perfringens* in broilers. The Avian Immunology Reseach Group Meeting, Guelph, Canada. Oral presentation.

KM Russell, P Kaiser, NH Sparks, MA Mitchell, RE Clutton, T Kanellos, S Athanasiadou (2013) Investigating host responses and disease pathogenesis in the duodenum of broilers exposed to crude *Clostridium perfringens* toxin *in situ*. BSAS and AVTRW, Innovation from Animal Science-a necessity not an option, Nottingham, UK. Abstract 195. Oral presentation.

KM Russell, P Kaiser, NH Sparks, S Athanasiadou (2012) Temporal responses in the duodenum of broilers exposed to crude *C. perfringens* toxin *in situ*. The Avian Immunology Research Group meeting, Edinburgh, UK. Poster presentation.

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List of abbreviations

Commonly used abbreviations throughout this thesis are given here. Others used will be identified in the text when used.

bp= base pairs
CSN= culture supernatant
DAMPs= Damage associated molecular patterns
FCR= Feed conversion ratio
IEL= Intra-epithelial lymphocytes
IFN= Interferon
IHC= Immunohistochemistry
IL= Interleukin
LMH= Liver male hepatocyte
LP= Lamina propria
MHC= Major histocompatibility complex
NE= Necrotic enteritis
NO= Nitric oxide
PAMPs= Pathogen associated molecular pattern
PCR= Polymerase chain recation
PRR= Pathogen recognition receptor
qPCR= Quantitative polymerase chain reaction
SE= Standard error
SN= Supernatant
TNF= Tumor necrosis factor
TLR= Toll-like receptor

Chapter 1 : General Introduction

1.1 Background

Necrotic enteritis (NE) is a major poultry disease that has a detrimental effect on profitability in the broiler industry. Previously, antibiotics added to broiler diets to increase weight gain and feed efficiency were believed to have minimised instances of NE (Long and Truscott, 1976; Prescott et al., 1978). Over the last couple of decades, there has been an increased pressure to reduce the use of antimicrobials in meat production. The European Union implemented Regulation (EC) No. 1831/2003 in 2006 to prohibit the use of in-feed antimicrobial growth promoters. The Food and Drug Administration (FDA) recently announced that it will implement legislation called the Veterinary Feed Directive (*Department of Health and Human Services*, 2015). This legislation will ensure that the use of antimicrobials, which are classed as being important for human health, are completely prohibited for production purposes and require veterinarian authorisation for control and treatment of disease. Large scale businesses are choosing to source meat from animals raised without antimicrobial input, which puts pressure on poultry producers to reduce the use of in-feed antimicrobials in their systems. In the broiler meat industry, this puts an increased stress on the intestinal health of chickens and is thought to cause a rise in the prevalence of diseases such as necrotic enteritis (Immerseel et al., 2004). After the withdrawal of avoparcin as an antibacterial feed additive in Norway, a spike in NE cases was observed (Grave et al., 2004). This was reduced to levels similar to those before the avoparcin ban following the approval of narasin. This drug was approved for use as an ionophore feed additive which inhibits the *Eimeria* parasite.

Broilers undergoing a drug-free programme in Canada had a reduced live weight at slaughter, reduced daily weight gain and increased feed conversion ratio (Gaucher et al., 2015). The drug-free program used essential oil alternatives rather than in-feed antimicrobials and anticoccidiostats which were used in the conventional systems. Zero percent of flocks raised in the conventional system had cases of NE whereas 27% of drug-free flocks had clinical NE and 49% had subclinical NE (Gaucher et al., 2015). These results support the idea that removal of these feed additives increases cases of intestinal disorders, specifically NE, which is estimated to cost the poultry industry around $2 billion annually (Sarson et al., 2009).

1.2 *Clostridium perfringens*

NE in broilers is caused by the Gram-positive bacterium, *Clostridium perfringens*. *C. perfringens* is an anaerobic bacterium which has the ability to form resistant endospores, allowing it to persist in decaying organic matter and soil (Matches et al., 1974). There are five known toxinotypes of *C. perfringens* named Type A-E based on their ability to produce a variety of toxins which play a part in disease pathogenesis (Hassan et al., 2015). Type A *C. perfringens*, named after its ability to produce large quantities of alpha-toxin, is the strain most commonly associated with NE (Cooper et al., 2010). Type B-E *C. perfringens* are classified on their ability to produce the Beta, Epsilon and Iota toxins as in Table 1.1.

Table 1.1. Major toxins used for toxinotyping *C. perfringens* isolates

C. perfringens Type	Toxin			
	Alpha	Beta	Epsilon	Iota
A	++	-	-	-
B	+	++	+	-
C	+	++	-	-
D	+	-	++	-
E	+	-	-	++

++ = Predominant toxin produced by type. + = Smaller quantities produced. - = Not produced by type. (Niilo, 1980).

Until 2006, alpha-toxin was considered the main virulence factor of NE but it has since been shown that mutant isolates without the alpha-toxin gene can also produce NE in broilers (Keyburn et al., 2006). This work highlighted the involvement of other unknown toxins and NetB was discovered soon after (Keyburn et al., 2008). NetB toxin is now considered to be the main virulence factor for NE in broilers, although other factors, including alpha-toxin, may still have a role in disease pathogenesis. These factors include Perfrin, TpeL and the Mu toxin.

Alpha-toxin isolated from *C. perfringens* was shown in early work to have enzymatic activity, meaning it can catalyse reactions at its site of action (MacFarlane and Knight, 1941). Alpha-toxin possesses phospholipase C and sphingomyelinase activity to degrade lipids in the cell membrane (Sakurai, 2004; Titball et al., 1999). The molecule is made up of N and C-domains, both of which are required to lyse cells (Uppalapati et al., 2013). Alpha-toxin binds with GM1a, a ganglioside on the cell membrane (Oda et al., 2012a). This induces an accumulation of diacylglycerol molecules in the cell

membrane which leads to activation of tyrosine kinase A (Oda et al., 2012a; Takagishi et al., 2015). Activation of tyrosine kinase A induces the release of interleukin-8 (IL-8) from a human alveolar basal epithelial cell line (Oda et al., 2012b). The action of alpha-toxin produces a source of cholesterol for the binding of another toxin, perfringolysin O (PFO), also known as theta-toxin (Moe and Heuck, 2010).

PFO is a member of the cholesterol-dependent cytolysin family. Members of this toxin family share 40-80% of their structural identity and have similar biological properties (Popoff, 2014). PFO is also part of a toxin family known as the thiol-activated cytolysin (TACY) family which are produced by Gram-positive bacteria and may work to synergize the effects of alpha-toxin (Billington et al., 2000). 40-50 monomer sub-units of PFO, produced by *C. perfringens*, oligomerize on the cell surface, where there is a source of cholesterol, and then insert a transmembrane domain creating a pore (Shepard et al., 2000). This allows for the passage of ions and macromolecules in and out of the cell (Billington et al., 2000). PFO from *C. perfringens* Type A has been shown in experimental models of gas gangrene to destroy host tissue and inflammatory cells in the area. As the toxin spreads it can diffuse into the systemic circulation where adhesion molecules are altered on polymorphonuclear leukocytes. This is thought to cause vascular leukostasis and regional tissue hypoxia (Bryant et al., 1993).

NetB (necrotic enteritis toxin B-like) is another pore-forming toxin produced by some isolates of *C. perfringens* Type A. The name NetB is given because of the similarity to *C. perfringens* β- toxin (Keyburn et al., 2008). Broilers

infected with an alpha-toxin mutant were NE positive after experimental challenge, indicating that this toxin was not solely responsible for virulence in the chicken (Keyburn et al., 2006). The discovery of NetB has initiated novel lines of enquiry into NE in broilers. This toxin has been identified in a number of field cases (Chalmers et al., 2008; Engström et al., 2012; Johansson et al., 2010; Martin and Smyth, 2009; Smyth and Martin, 2010). NetB has a limited sequence similarity to other pore-forming toxins but does share sequence identity with the β- and δ-toxins from *C. perfringens* (38 and 40%, respectively) as well as α- and γ-hemolysin from *Staphylococcus aureus* (Savva et al., 2013). Seven subunits of NetB come together at the cell membrane to form a pore which is similar to the assembly and action of α-hemolysin (Savva et al., 2013). NetB is enhanced when there is a source of cholesterol present but the receptor this toxin utilises to bind with the cell is still unknown (Popoff, 2014).

The percentage of isolates containing the NetB toxin (in healthy and infected birds) varies between countries. In Australia, 70% of *C. perfringens* isolated from diseased birds have been shown to possess the NetB gene and all of these isolates produced NetB *in vitro* (Keyburn et al., 2010) . In the United States, 58% of isolates from birds with NE and 9% of isolates from healthy birds were positive for NetB toxin (Martin and Smyth, 2009). Similar results were seen in Canada, where 95% of the isolates from diseased birds contained the NetB gene. However, 35% of isolates from healthy birds also contained the NetB gene (Chalmers et al., 2008). A study in Denmark showed that 61% of *C. perfringens* isolates from healthy birds contained NetB. Prevalence of

the NetB gene in diseased birds was also low, with 52% of the isolates tested containing the gene. The presence of the gene does not necessarily mean that the bacteria will produce NetB, as not all isolates positive for NetB gene produced the toxin *in vitro* (Abildgaard et al., 2010). Isolates from diseased birds were, however, were more likely to produce this toxin than isolates from healthy birds (Abildgaard et al., 2010).

C. perfringens has been shown to contain three pathogenicity loci which may contribute to NE pathogenesis. Locus 1, NELoc1, is located on a plasmid which holds the NetB gene and is approximately 42kb in length. The other two loci, NELoc2 and NELoc3, are shorter at 11.2 and 5.6kb respectively. NELoc3 is also located on a plasmid but NELoc2 is chromosomally located. Seven different isolates of *C. perfringens,* which caused NE, were shown to have these three pathogenicity loci (Lepp et al., 2010).

A recently discovered bacteriocin, Perfrin, has been described as a possible virulence factor for NE. Bacteriocins are molecules which have antibacterial properties (Nishie et al., 2012). The gene encoding this antimicrobial protein was present on a number of NetB-positive isolates but not found on NetB negative isolates. Perfrin has bacteriocidal activity against other *C. perfringens* isolates. It exerts this activity on all isolates without the perfrin gene meaning it could be important in promoting the growth of poultry virulent isolates which possess it (Timbermont et al., 2014).

Other toxins produced by *C. perfringens* may facilitate pathogenesis of NE such as the TpeL toxin which was originally found in *C. perfringens* Type C but has also been found in Type A. It is a member of the large clostridial

cytotoxins (LCTs) which have been shown to increase the severity of NE. These toxins have at least four active domains, “ABCD”, where the B domain binds to the cell. The toxin is endocytosed and the D domain inserts into the endosome membrane. Components of the cytosol activate the protease C domain which results in cleavage of the toxin and release of the A domain. The A domain has the ability to inactivate GTPases in the cytosol. TpeL modifies Rac1 and Ras to mediate its cytotoxic effects (Coursodon et al., 2012; Timbermont et al., 2011). These small GTPases have roles in actin cytoskeleton reorganization and cell proliferation respectively (Nagahama et al., 2011). A Mu toxin can also be produced by Type A strains of *C. perfringens*. It is a hyaluronidase and degrades hyaluronic acid in the extracellular matrix. It is thought to increase the virulence of *C. perfringens* by potentiating the effects of other toxins through increasing cellular permeability (Adams et al., 2008; Canard et al., 1994).

The enterotoxin present on some isolates of *C. perfringens* is associated with gastrointestinal disease in humans. This toxin binds with claudin molecules which are components of tight junctions (Veshnyakova et al., 2012). Enterotoxin is another pore-forming toxin and forms a pre-pore on the cell surface prior to inserting into the cell (Gao and McClane, 2012). The formation of these pores allows calcium entry into the cell. At low levels this induces apoptosis of the cell and at higher levels this can cause oncosis, where cells increase in volume and induce inflammatory cell death (McClane and Chakrabarti, 2004).

During NE diagnosis confirmation of the isolate type is required. To ensure correct typing of *C. perfringens* Type A the absence of the other typing toxins should also be confirmed. As indicated in Table 1.1, *C. perfringens* β-toxin is present on Type B and C isolates. This pore-forming toxin, like NetB, has similarities to other toxins such as *S. aureus* alpha-toxin with 28% sequence similarity (Nagahama et al., 2015a). β-toxin is associated with necrotising enteritis in a variety of agricultural species and humans (Uzal and McClane, 2011). Epsilon-toxin is essential for Type D isolate virulence in sheep, goats and mice (Garcia et al., 2013) but can also be present on Type B isolates (Alves et al., 2014). This is considered to be one of the most virulent bacterial toxins produced (Alves et al., 2014). Iota-toxin is a member of the binary toxin family and is comprised of two separate polypeptides, IA and IB (Rood, 1998). These proteins are secreted as pro-molecules and require activation via proteolytic cleavage of the N-terminal region. IB interacts with host cell lipoproteins. Once bound, IA interacts with IB to promote endocytosis of iota-toxin. In turn the cytoskeleton of the host cell collapses (Moore et al., 2014).

The activation of many virulence genes within *C. perfringens* and the release of virulence factors are controlled by a two component signal transduction system. This consists of a sensor molecule, VirS, and a responder molecule, VirR. VirS is a trans-membrane protein. The extracellular domain senses the external environment out with the cell and promotes auto-phosphorylation of the intracellular domain. This, in turn, causes the phosphorylation of VirR in the cytoplasm. NetB is regulated by the VirSR system. It is produced after 4

hours inoculation in the late logarithmic phase of bacterial growth (Cheung et al., 2010). The genes encoding for VirSR were originally discovered in their role of regulating perfringolysin O, alpha-toxin and sialidase release but they also regulate a number of genes involved in macromolecule degradation, which provide nutrients for the bacteria. Other genes which appear to be controlled via this system are involved in nutrient import and metabolising. (Lyristis et al., 1994; Ohtani et al., 2010; Shimizu et al., 1994). It remains unclear what activates VirS to produce the release of virulence factors but preventing toxin release could prevent the necrotic damage caused in NE.

1.3 Necrotic enteritis pathogenesis

Broilers develop NE approximately three-four weeks after hatching (Engström et al., 2003; Lovland and Kaldhusdal, 2001). The disease can present in either clinical or sub-clinical forms. The clinical form can cause death in 1-2 hours with birds showing signs of depression. (Helmboldt and Bryant, 1971). At post-mortem, large parts of the intestine are necrotic and covered with a yellow-brown pseudomembrane that is filled with necrotic cells, bacterial colonies and tissue fragments (Lee et al., 2011; Timbermont et al., 2011). Outbreaks can be controlled with antibiotics.

Sub-clinical NE is thought to be more of a problem for the poultry industry as it may persist in flocks without presenting any clinical signs. Birds, therefore, go un-treated with antibiotics and bacteria may enter the food chain, which may lead to food poisoning in humans (Immerseel et al., 2004). 10% of all foodborne diseases in the USA are thought to be caused by *C. perfringens* (Scallan et al., 2011). Furthermore, birds with subclinical NE often show a

reduced weight gain due to an inability to digest and absorb feed, leading to a decreased feed conversion ratio (FCR). Various studies looking at dietary interventions for NE treatment have measured FCR. Infected birds often have around a 10% increase in FCR compared with un-infected controls (Skinner et al., 2010). Examples of increased FCR are shown in Table 1.2.

Table 1.2. Examples of reduced feed conversion in broilers challenged with *C. perfringens*

Bird age at challenge (day of life)	*Eimeria* (Y/N)	Control group FCR	NE group FCR	% increase of FCR in NE birds	Significant difference (Y/N)	Ref
17-20	N	1.74	1.82	8%	~ (p=0.064)	(Liu et al., 2010)
0	Y	1.377	1.388	1.1%	Y	(Cravens et al., 2013)
19-21	Y	1.54	1.60	6%	Y	(Jayaraman et al., 2013)
14-15	~	1.77	1.90	13%	Y	(Rodgers et al., 2015)
14	Y	1.268	1.346	9.2%	Y	(M'Sadeq et al., 2015)

Subclinical NE can also be associated with a deterioration of the litter material increasing the risk of food pad dermatitis and hock burn; two conditions that are large welfare problems for the industry (Allain et al., 2009). At post-mortem, ulcers are typically seen in the form of a depression

on the mucosal surface covered with a loosely adherent yellow or greenish material (Figure 1.1).

Figure 1.1. Gross NE lesions

Broiler intestine of birds challenged with *C. perfringens* type A. **A-**gross lesions after challenge with a NetB positive strain of *C. perfringens*. **B**-the intestine of a broiler challenged with a NetB negative strain which looks healthy (Cooper and Songer, 2009).

In the sub-clinical form of the disease, the bacteria have the ability to transfer and colonize the liver, via the bile duct, to cause cholangiohepatitis and ascites, an accumulation of fluid in the peritoneal cavity (Kaldhusdal et al., 2001; Olkowski et al., 2006). Birds which have *C. perfringens* associated lesions in the liver can be condemned at slaughter (Løvland and Kaldhusdal, 1999; Lovland et al., 2003). Histologically, the lamina propria of the gut becomes hyperaemic but the epithelium is relatively normal in experimental birds with subclinical NE (Olkowski et al., 2006). Lymphocytes, granulocytes,

macrophages, plasma cells and some eosinophils infiltrate the lamina propria (Figure 1.2). At the site of interaction, the basal domain, enterocytes and the lamina propria become oedematous. Villi are shortened and crypts become distended. Necrosis of epithelial cells can be characterised by chromatin condensation, karryorrhexis and karyolysis (Engberg et al., 2002). The financial cost to the industry comes from the extra feeding required due to reduced efficiency, the housing of birds that will be condemned at slaughter and any treatment required to restore health in the flock.

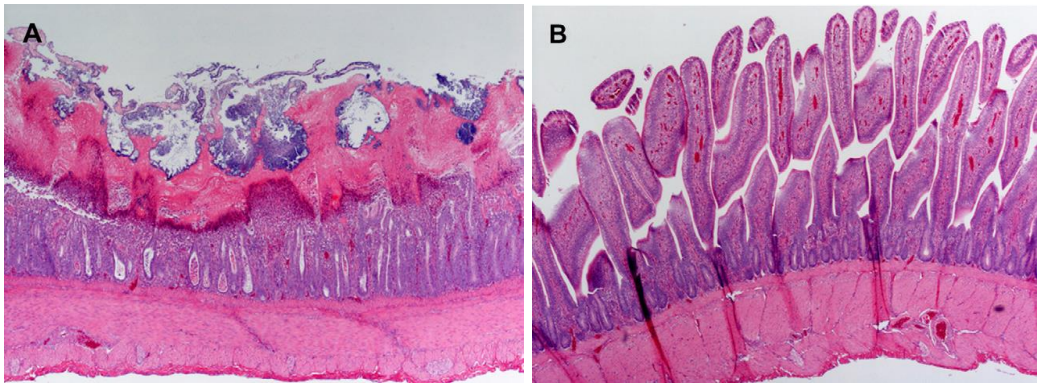

Figure 1.2. Haematoxylin and eosin sections from a field case of NE
Images of H&E stained sections from a bird infected with a NetB positive strain of *C. perfringens* Type A from a field case of NE (**A**) and a bird infected with a NetB negative strain of *C. perfringens* Type A (**B**). The crypts are distended and there is immune infiltration in **A** but no microscopic lesions are seen in **B** (Cooper and Songer, 2010).

Although the disease pathogenesis is not fully understood, it appears that *C. perfringens* antigens and toxins alone are not adequate to cause the disease. *C. perfringens* Type A has been found in the intestinal microbiota of

apparently healthy animals but only when the poultry virulent bacterial strains are combined with other pre-disposing factors does it manifest into NE (Cooper and Songer, 2010). Diet composition appears to affect NE pathogenesis; for example the inclusion of large amounts of cereals, which are often rich in water soluble non-starch polysaccharides (NSP) predispose NE. Increasing concentrations of carboxymethyl cellulose (CMC), a NSP, increases viscosity in the gut and allows time for an increase in bacterial activity (Branton et al., 1997). Broilers fed a wheat-based diet and a wheat based diet supplemented with complex carbohydrates and additional fibre produced more lesions than those given a corn diet. (Branton et al., 1997).

The presence of undigested protein in the lower gut of broilers has been associated with NE. The percentage of protein in the diet and also the nature of the protein has been linked with NE outbreaks (Fernando et al., 2011; Palliyeguru et al., 2011). Fernando et al. (2011) showed that birds on a potato protein diet (which is poorly digested) produced a higher titre of alpha-toxin antibody, had a significant increase in hepatic lesions and a higher mean incidence of intestinal necrosis compared with birds fed a soya bean protein diet. Protein from an animal source has also been implicated in pre-disposing NE. *C. perfringens* counts from the ileum and caecum were higher in broilers fed a fishmeal-based diet and counts also increased when crude protein levels were increased (Drew et al., 2004). The quality of protein in this diet is high and is not always fully digestible, allowing for some to pass to the lower gastrointestinal tract. Different protein sources can have different concentrations of amino acids. Some amino acids may stimulate growth of *C.*

perfringens and, therefore, contribute to NE incidence. Increasing numbers of *C. perfringens* cells are detected in the ileum and caecum when increasing levels of glycine are included broiler diets (Palliyeguru et al., 2011). Feeding with a fishmeal protein source has the ability to alter the dynamics of the broiler intestinal microbiota which may create the correct conditions for *C. perfringens* to colonise the intestine (Stanley et al., 2014; Wu et al., 2014) .

The form of diet offered to broilers has also been implicated with NE pathogenesis. Engberg et al. (2002) found decreased *C. perfringens* counts when broilers were fed a diet in pellet form when compared with a mash diet but this study did not score for intestinal lesions. Feed restriction is also likely to change the intestinal ecosystem. Broilers infected with *Eimeria* (10x Paracox vaccine) and *C. perfringens* fed *ad libitum* had increased lesion scores in comparison to birds which were feed restricted for twelve hours per night during the infection protocol (Tsiouris et al., 2014). Feed restricted, infected broilers had significantly reduced intestinal pH and cecal *C. perfringens* counts compared to their *ad libitum* counterparts. The mechanisms for reduced lesions scores in restricted fed birds were unclear but it was hypothesised that increased levels of glucocorticoids caused by the feed restriction may in turn reduce levels of prostaglandins and suppress inflammation (Tsiouris et al., 2014).

The environment can also play a factor in the predisposition of birds to NE. A survey of broiler farms in the UK showed that farms with wet litter, plaster board walls or those that used ammonia as a disinfectant were found to have a higher prevalence of NE (Hermans and Morgan, 2007). Broilers undergoing

heat stress during the infection protocol had lower lesion scores than those that were infected without heat stress (Calefi et al., 2014) but temperature stress may also act as a predisposing factor prior to infections (Lee et al., 2011). Increased stocking density has also been implicated as a predisposing factor which reduces broiler welfare and impacts on gut health to favour NE development (Tsiouris et al., 2015).

Other pre-disposing factors that have been described include host immunosuppression, which may also influence disease incidence (Casterlow et al., 2011). A bird's immune system may become suppressed during a co-infection with another micro-organism or virus. A co-infection with *Eimeria maxima* is commonly associated with NE incidence in broilers. It is often used in experimental models of NE with *C. perfringens* to produce NE lesions as it alters the microbiota composition (Stanley et al., 2014; Wu et al., 2014). *E. maxima* is an intracellular protozoan parasite that causes coccidiosis. This parasite produces intestinal lesions and causes destruction of the intestinal epithelium during the intracellular stages of its life cycle. Plasma proteins leak into the lumen providing a source of nutrients for *C. perfringens* (Collier et al., 2008). It has been shown that animals with *E. maxima* infections have a reduced expression of liver expressed anti-microbial peptide-2 (LEAP-2) (Casterlow et al., 2011). This may help NE causing *C. perfringens* to proliferate within a gut that has already been damaged. In an experimental model of NE with *Eimeria* and *C. perfringens* co-infection, the immunosuppressive cytokine, IL-10, was found to be significantly increased but with *C. perfringens* infection alone no changes

were detected suggesting the parasite may allow a greater opportunity for *C. perfringens* to proliferate (Park et al., 2008). Other immune mediators such as IFN-α, IFN-γ and IL-1β are all down-regulated in co-infection models when compared with *C. perfringens* alone in the days after infection. IL-8 is up-regulated along with IL-10 (Parvizi et al., 2010).

Immunosuppressive viruses, such as Marek's disease virus, infectious bursal disease virus (IBDV) and chick anaemia virus (CAV), may also play a part in the development of NE (Lee et al., 2011; McReynolds et al., 2004). Marek's disease virus causes cytolysis of B cells and T cell transformation which leads to immunosuppression and lymphomas (Müller et al., 2003). IBDV targets lymphoid cells in the Bursa of Fabricius resulting in lymphoid depletion and immunosuppression in birds leaving them open to other infections (Hailemariam et al., 2008). Anaemia, bone marrow aplasia, thymus atrophy and immunosuppression characterise the disease known as chick infectious anaemia caused by chick anaemia virus (Flores-Diaz et al., 2005). The losses initiated by subclinical NE can be substantial to poultry producers. Gross and microscopic lesions are well described in the literature but it is clear that various factors influence disease onset during the development of NE. There is still much to be learned about the role of these various factors before a standardised infection model can be introduced.

1.4 Broiler Immune Response

Until recently the use of in-feed antimicrobials and anticoccidiostats were thought to have prevented NE from becoming a significant problem for the poultry industry, so the immune response to *C. perfringens* in the chicken

has not been well characterised. Similar to mammals, chickens have both the general arms of innate and adaptive immunity. There are, however, differences in the avian system. Mainly the avian system appears to have fewer receptors and effector molecules than the mammalian system. Innate immunity provides an early line of defence against pathogens and consists of antigen recognition receptors, phagocytic cells and secreted barrier molecules. On the other hand, the adaptive response is pathogen specific and mediated by T cells and B cells.

Table 1.3. Components of the innate and adaptive immune system

	Innate Immunity	Adaptive Immunity
Cells	Heterophils Macrophages Dendritic cells	CD4+ T cells CD8+ T cells Regulatory T cells γδ T cells B cells
Cytokines	IL-1ß, IL-6, IFN-α, IFN-ß	IL-12, IL-18, IFN-γ, IL-4, IL-13, IL- 10
Cell receptors	Toll like receptors e.g. TLR2.1, TLR2.2, TLR4, TLR21 Mannose receptor Scavenger receptor	T-cell Receptor Ig Receptor
Secreted components	Mucins B-defensins (1-14)	Antibody e.g. IgY, IgM, IgA

In relation to NE the immune system in the intestine is of interest. The chicken gut develops rapidly in the last few days prior to and just after hatching for nutrient digestion and absorption. After hatching, the gut quickly becomes colonised with bacteria and it must adapt to balance digestive functions with protecting the host from pathogens. Initial protection arises from physical measures, which actively inhibit pathogen attachment to the epithelium, and chemical measures which have the ability to disrupt microbial cell membranes. Microbial colonisation of the intestinal

tract in birds is required for the development of the immune system. Birds kept in a germ-free environment have poorly developed lymphoid follicles in the cecal tonsil with no IgG or IgA positive cells detected at four weeks of age in comparison to their conventionally housed counterparts. Germ free birds also had fewer T cells (CD3+) in the villus regions of the cecal tonsil compared with the conventional birds (Honjo et al., 1993). The expression of the CD3 gene, which is a marker for T cells, is detected at low levels in the first days of life but increases substantially at day four, indicating increased development of the T cell population in time throughout the intestine (Bar-Shira et al., 2003). The intestinal immune system must develop to distinguish between commensal and pathogenic bacteria so that effective responses can clear organisms likely to invade and destroy host tissues.

1.4.1 Innate responses

1.4.1.1 Barrier molecules

One of the first lines of defence in the chicken intestine is the mucin barrier and these mucin molecules are likely to be some of the first molecules *C. perfringens* will come in contact with. Eight mucin genes have been identified in the chicken genome. Five of these are secreted proteins (Muc2, Muc5ac, Muc5b, Muc6, Ovomucin) and three are transmembrane molecules (Muc13, Muc16 and Muc1) (Lang et al., 2006). Secreted mucins are predominantly released from goblet cells. These cells are part of the epithelial layer that separate the lumen from the lamina propria, which produce a mucous layer that is predominantly made up of mucin glycoproteins. These can be released by baseline secretion or compound exocytosis (Kim and

Khan, 2013). Baseline secretion is the continuous release of mucin molecules and compound exocytosis is the release of central mucin stores after stimulations from hormones, neuropeptides and inflammatory mediators. Alternatively, transmembrane mucins are found on the surface of enterocytes. These molecules form part of the glycocalyx, a region at the apical end of enterocytes which prevents bacterial attachment (Pelaseyed et al., 2014). Muc13 has been identified in the chicken and the structure identified indicates this molecule can be produced in larger and smaller lengths providing a barrier at different regions from the cell surface (Lang et al., 2006).

Mucins as part of the mucous layer prevent damage from the contents of the lumen as well as stopping the adherence of pathogens to the intestine wall. Its composition can be altered by nutrients and antimicrobial compounds. Crude mucin increases with the inclusion of increasing amounts of threonine in the diet (Chee et al., 2010; Horn et al., 2009). *In ovo* administration of mannan oligosaccharide (MOS) 3 days prior to hatching increased the levels of Muc2 before hatching (Cheled-Shoval et al., 2011). Similar results are also seen in broilers fed a diet supplemented with MOS. Muc-2 is generally expressed at higher levels in birds supplemented with MOS when compared with birds without in the jejunum (Chee et al., 2010). Some studies have investigated mucin mRNA expression after NE challenge and detected variations in Muc2, Muc5ac and Muc13 transcripts in the days post-infection but these are not always consistent between studies. Collier et al., 2008 measured increased Muc2 mRNA expression in the ileum of birds challenged

with *C. perfringens* and a co-infection with *Eimeria* on the final day of infection and two days later. Forder et al., (2012) detected increased Muc5ac mRNA three days after an NE co-infection challenge and reduced Muc2 and Muc13 mRNA expression at the same time point. Kitessa et al., (2014) found changes in Muc5ac and Muc13 expression with pre-disposing factors but mRNA levels were similar to controls when *C. perfringens* was added to the experimental challenge. It is possible that these inconsistencies could be attributed to differences in the challenge models as all three inoculate the birds on different days, not all use the same pre-disposing factors and there could also be differences in mucin expression between regions of the small intestine.

Other defence molecules present in the avian intestine are the β-defensins. These are antimicrobial peptides which are produced by heterophils and are also found throughout other tissues in the bird from early in development (Meade et al., 2009). Fourteen genes for β-defensins have been identified in the chicken genome, AvBD1-14, so far (van Dijk et al., 2008). The transcriptional profile is different for each one depending on which pathogen and which tissues are involved (Cuperus et al., 2013; van Dijk et al., 2008). A co-infection model in Ross and Cobb broilers with *E. maxima* and *C. perfringens* showed altered expression of defensin genes in the crop and jejunum (Hong et al., 2012). There were few changes in the defensin levels detected in the crop between the infected birds and uninfected controls. AvBD1, 6 and 7 mRNA levels were increased in infected Cobb broilers while AvBD11 was reduced two days post infection. Infected Ross broilers had

increased AvBD2 and reduced AvBD6 in comparison to uninfected controls at the same time point. In comparison there were more changes in AvBD mRNA expression in the jejunum. AvBD8 was the only defensin where increased mRNA was detected in infected broilers of both breeds and AvBD12 was reduced in both. AvBD8, 10 and 13 were highly expressed in the jejunum, however, the expression of AvBD13 was not significantly higher in infected broilers compared with control birds. Greater levels of these β-defensins were seen in Ross broilers when compared to Cobb broilers which may imply some genetic differences in ability to mount an immune response in NE (Hong et al., 2012).

1.4.1.2 Intestinal Epithelial and Immune Cells

Pathogens which are able to disrupt the mucous barrier and evade these antimicrobial peptides will then interact with the epithelium of the chicken intestine. Goblet cells, enterocytes and intra-epithelial lymphocytes (IEL) are some of the cells that make up the epithelial layer (Brisbin et al., 2008). The IEL populations are comprised of NK cells, T cells and B cells (Gobel et al., 2001). Interactions with the epithelium can activate pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs), and their pathways. PRRs are found on various cell types such as dendritic cells, heterophils and endothelial cells. TLRs are important in the recognition of pathogen associated molecular patterns (PAMPs) which are found on the surface of bacteria. These molecules on the surface of cells trigger pathways which up-regulate the expression of inflammatory molecules such as cytokines and chemokines that attract increased numbers of inflammatory cells to the site

of infection (Kaiser, 2010). TLR genes are expressed throughout the intestine which may reflect the wide array of pathogens that can be detected in this tissue (Brownlie et al., 2009; Iqbal et al., 2005). These molecules detect various components of pathogens, including bacterial lipopeptides, double and single stranded RNA. A summary of the TLRs present in the chicken and their activating ligands are shown in Table 1.4.

Table 1.4. Toll-like receptors with their typical TLR ligands in humans and chicken

Ligand	TLRs in humans	TLRs in chickens
Triacylated lipopeptides	TLR2/TLR1	TLR2.1/TLR1.1, TLR2.1/TLR1.2, TLR2.2/TLR1.1
Diacylated lipopeptides	TLR2/TLR6 TLR2.2/TLR16	TLR2.2/TLR1.1, TLR2.1/TLR1.2
Triacylated lipopeptides (possibly)	TLR2/TLR10	Absent
dsRNA	TLR3	TLR3
LPS	TLR4/MD-2	TLR4/MD-2
Flagellin	TLR5	TLR5
ssRNA	TLR7	Possibly TLR7
ssRNA	TLR8	Not functional
DNA	TLR9	Absent
DNA	Absent	TLR21
Protease	Absent	TLR15

Summarised from Keestra et al., 2013. TLRs separated with / form heterodimers to become functional pathogen recognition receptors.

TLR gene expression in the ileum has been shown to alter in the first few days after *C. perfringens* challenge on day 18 of life (Lu et al., 2009). Changes in TLRs are also detected later in challenge models with differences being described one week after *C. perfringens* challenge (Cao et al., 2012; Yitbarek et al., 2012). TLR4 mRNA was not differentially expressed in either experiment in the ileum or cecal tonsil. Yitbarek et al., (2012), detected increased TLR2.2 in the cecal tonsil but not in the ileum. Conversely, TLR2.2 mRNA levels increased after *C. perfringens* challenge in the ileal mucosa in a separate study (Cao et al., 2012). Again, differences in the detection of TLR genes between these different studies could in part be related to the levels of

challenge given to the broilers and the time between the *C. perfringens* challenge and sampling.

In addition to epithelial cells, a number of immune cells also express TLRs, including heterophils, macrophages and IELs. These cells have different functions during host responses to bacterial infections. Heterophils are part of the innate response, are the avian equivalent of neutrophils and are located in the lamina propria of the small intestine. They are polymorphonuclear cells that phagocytose invading pathogens. Once pathogens have been internalised by a heterophil they can be killed by a respiratory burst or degranulation (Kogut et al., 2006). The respiratory burst involves NADPH oxidase being activated to produce superoxide which in turn is converted to hydrogen peroxide. This is converted to hypochlorous acid which is thought to have bactericidal activity. Degranulation refers to the killing of microbes by the release of proteins into the phagosome (Genovese et al., 2013). Activation of different TLRs on heterophils produces different cytokine and chemokine responses. Also, heterophils from genetically different broiler lines vary in their responses to TLR activation (Kogut et al., 2006). Early exposure to certain bacteria may improve heterophil responses in broilers. Heterophils from broilers given probiotics on the day of hatch had improved oxidative burst and degranulation responses than birds which did not receive the probiotic treatment (Farnell et al., 2006). Heterophils infiltrate NE lesions in the intestine but it is unclear what role they play in combatting the disease (Shane et al., 1985). This is different from other *C. perfringens* infections,

such as gas gangrene, where alpha-toxin appears to prevent the chemotaxis of neutrophils to the site of infection (Flores-Dı´az and Alape-Girón, 2003).

1.4.2 Bridging Between Innate and Adaptive Immunity

Like heterophils, γδ T cells present in the intestine are also able to respond to direct contact with bacterial cells; αβ T cells on the other hand, require interaction with antigen presenting cells (Bennett et al., 2015; Gao and Williams, 2015). Intra-epithelial γδ T cells in mammals are important for preventing bacterial invasion in the intestine (Edelblum et al., 2015). Once activated this cell type can directly kill infected target cells, promote maturation of dendritic cells and interact with αβ T cells and B cells (Vantourout and Hayday, 2013). γδ T cells also reside within the lamina propria but cells in these two locations play different roles in the innate immune response. Those in the intra-epithelial layer are IFN-γ producing cells and those in the lamina propria produce IL-17 in response to pathogens (Vantourout and Hayday, 2013). In mammals, IL-17 producing γδ T cells have the ability to increase in numbers in response to pathogens (Martin et al., 2009). Characterisation of γδ T cell populations in the chicken has been performed and it has been shown that the presence of CD8 markers can change after infection (Pieper et al., 2011, 2008). Expression of the T cell receptor γ- chain was increased in the spleen of broilers in the first four days after *C. perfringens* challenge indicating that γδ T cells may play a role in response to this pathogen. No information exists in relation to *C. perfringens* interactions with γδ T cells in the intestine of broilers. Further work is

required to determine whether γδ T cells could have a protective response during NE and how early they are effective in eliciting this response.

Some recent work has investigated transcriptional changes in intra-epithelial lymphocytes (IELs) after co-challenge with *E. maxima* and *C. perfringens* with the aim of further elucidating how these immune cells respond during NE disease progression. IELs from infected Ross broilers differentially expressed genes with functions related to leucocyte cell movement, quantity of leukocytes and immune response (Kim et al., 2014). Genes which were differentially regulated in the infected Ross broilers were also investigated in IELs from two lines of in-bred birds which have differing disease susceptibility. The susceptible line 6.3 had increased expression of 15 immune related genes in comparison to the resistant line 7.2 (Kim et al., 2014). MicroRNA from IELs in these two in-bred lines was differentially regulated. MicroRNA can prompt gene silencing by inhibiting mRNA translation or causing target gene degradation. The NE resistant line 7.2 expressed increased levels of microRNAs than the 6.3 susceptible line (Hong et al., 2014). The role these microRNA and their target mRNA in disease progression is still not understood. It is also unclear which cells are predominant in the IELs of these two inbred lines. The differences detected may be attributed to a different balance of γδ T cells, NK cells and B cells in the intra-epithelial layer of each breed. Currently, it is not possible to investigate such relationships due to the lack of specific antibodies and protocols that can define the cell types and the pro-inflammatory mediators they produce.

IELs interact with other cells within the lamina propria to fight infection. *C. perfringens* reaching the lamina propria could be phagocytosed by antigen presenting cells such as macrophages and dendritic cells (de Geus and Vervelde, 2013; Klasing, 1998; Mast et al., 1998). As an infection develops, the innate and adaptive immune systems work in conjunction with each other to combat disease. During the acquisition of adaptive immunity, antigens are displayed to the adaptive immune system by MHC on their cell surface. Dendritic cells and other antigen presenting cells phagocytose pathogens and display antigen molecules on MHC Class II complexes (Roche and Furuta, 2015). CD4+ T cells recognise this complex to initiate an adaptive response. MHC Class I molecules found on all other cell types display antigens to CD8+ T cells (Neefjes et al., 2011). Expression of MHC Class I and II in the intestine has not been previously described in *C. perfringens* infection but transcriptomic analysis of the spleen of infected broilers has indicated changes in these antigen presentation complexes. B2-microglobulin, calnexin and calreticulin were significantly up-regulated in the spleen of *C. perfringens* infected chickens. These molecules are important in MHC Class I assembly and the processing and presenting of antigens to CD8+ T cells suggesting that these cytotoxic cells are involved in the response (Zhou et al., 2009). In the same study, genes for B-Lb and the invariant chain, two components of MHC Class II, were also up-regulated suggesting antigen presentation to CD4+ cells may also be important in *C. perfringens* infections. These results indicate that various T cell subsets are involved in the immune response to NE.

1.4.3 Cytokine responses

All the immune cells described previously have the ability to produce a number of cytokines and/or chemokines to attract cells to the site of infection and direct the immune response in a specific manner. IL-12, IFN-γ, IL-6 and IL-10 expression showed no changes in gene expression in the ileum and cecal tonsils one week after challenge with *C. perfringens* (Yitbarek et al., 2012). An increase in IL-4 production is detected two days after challenging birds with *C. perfringens,* on three consecutive days. This suggests a proliferation of the Th2 line of CD4+ T cells (Collier et al., 2003). IL-18 is up-regulated in the spleen of *C. perfringens* challenged broilers in the days post infection (Sarson et al., 2009). IL-18 induces a Th1 response from CD4+ T cells (Gobel et al., 2003). From the current literature, it is not clear at which stages these different cells may be involved and which is more critical in the clearance of virulent *C. perfringens*. The up-regulation of activated T cells may provide the help required for B cell differentiation and, therefore, antibody production. The chicken IgY and IgY receptor genes were up-regulated in the spleen following an experimental infection with *C. perfringens* showing that B cell antibody responses are induced during infection. A four-fold increase in the expression of the receptor gene was observed four days post challenge although a two fold increase was observed within the first two days post challenge (Sarson et al., 2009; Zhou et al., 2009).

Responses to *C. perfringens* Type A toxins alone have currently not been well characterised. Al-Sheikhly and Truscott showed that crude toxin alone from

C. perfringens Type A injected into the duodenum did reproduce intestinal lesions typical of NE. Histology from this work also showed some immune cell infiltration but this was not quantified (Al-sheikhly and Truscott, 1977a). Broilers may have variability in their responses to *C. perfringens* and its toxins depending on their genotype. Peripheral blood mononuclear (PBMNs) cells from birds genetically selected for high antibody (HA) or low antibody (LA) responses vary in how they react to alpha-toxin. These birds have a difference in major histocompatibility complex (MHC) haplotype. There were also a number of differences in cytokine expression after incubation with alpha-toxin from PBMNs in the first hours after toxin exposure (Sumners et al., 2012). Currently, it is not known if NetB exposure would result in similar effects in the cell population.

Recent work using toxin supernatant in vaccine development has had varying successes. Inoculation of birds with a NetB positive supernatant from an isolate which also produces low levels of alpha-toxin prevented the development of lesions in broilers when they were later challenged with *C. perfringens*. Supernatants from other isolates produced limited protection and therefore did not make good vaccine candidates (Lanckriet et al., 2010). It is currently not known whether *C. perfringens* toxins *per se* have an effect on the recruitment of immune cells or their activity in broilers during NE infections. As previously mentioned, NetB toxin from *C. perfringens* has a sequence identity similar to that of *Staphylococcus aureus* α-haemolysin. The absence of expression of α-haemolysin toxin during a rodent skin and soft tissue infection with *S. aureus* prompts the up-regulation of pro-

inflammatory cytokines and chemokines. These in turn result in an early influx of IL-17+ γδ T cells and swift activation of Th1 and Th17 adaptive cells. Wild type isolates with α-hemolysin suppress these protective responses (Tkaczyk et al., 2013). Improved characterisation of toxin supernatant and immune system interactions would increase the chances for the development of robust vaccines in the future.

There is evidence from *in vitro* experiments and mouse models that some of the toxins produced by *C. perfringens* could promote evasion of the host immune system. *C. perfringens* incubated with a murine macrophage cell line were present in the cytoplasm rather than the phagosome of the macrophages, permitting the bacteria to survive within the cell and not be processed for antigen presentation (O'Brien and Melville, 2000). It has been shown that this evasion is in part mediated by alpha-toxin and perfringolysin-O, as mutant *C. perfringens* which lack these genes do not survive in macrophages for the same length of time as wild type isolates. These toxins also contribute to persistence *in vivo* (O'Brien and Melville, 2004). As well as promoting this persistence in macrophages *C. perfringens* alpha-toxin also alters the response of these cells. A rodent macrophage cell line was incubated with alpha-toxin and then stimulated with LPS. The production of TNF-α and nitric oxide (NO) was lower in cells incubated with alpha-toxin when compared with cells that were not which implies that alpha-toxin alone has the ability to alter cellular immune responses (Tumurkhuu et al., 2009). Further investigation with NetB and other *C.*

perfringens toxins may provide insight into whether this bacterium has the ability to evade the chicken immune response.

1.5 Animal experimental models

The number of environmental factors as well as varying exposure to *C. perfringens* and its virulence factors has meant that various experimental challenge models have evolved in the literature.

The number of environmental factors as well as varying exposure to *C. perfringens* and its virulence factors has prevented a reliable animal disease model from being reproduced with each infection. In data that originate from field cases, birds suffering from NE contain larger numbers of *C. perfringens* in their intestines, between 10^6 and 10^8 colony forming units per gram (CFU/g) of intestine, when compared with non-diseased broilers which had up to $10^{4.8}$ CFU/g (Long et al., 1974). *Eimeria necatrix* infection as part of a NE challenge model induced higher numbers of *C. perfringens* in the first week after infection than challenge with the bacteria alone (Baba et al., 1997). When a variety of *C. perfringens* isolates were used to carry out experimental challenge only one could be recovered afterwards. This was a poultry virulent isolate from a case of poultry enteritis which actively inhibited growth of normal microflora isolates (Barbara et al., 2008). This indicated that some isolates of *C. perfringens* have a greater ability to colonise the chicken intestinal tract. Experimentally creating the correct conditions with predisposing factors to induce *C. perfringens* growth to the numbers described before has been a challenge for research groups. These results and

the discovery of new virulence factors have highlighted that the isolate used for experimental challenge can be important in reproducing the disease.

Variation occurs between isolates which have the NetB gene. *C. perfringens* culture supernatant produced from isolates characterised for the presence of NetB and alpha-toxin production did not all induce the same levels of cytotoxicity on the LMH cell line (Lanckriet et al., 2010). The NetB gene, which encodes for the toxin production is encoded on a plasmid (Bannam et al., 2011). This plasmid has a region called the tcp-locus, which is made up of 11 genes involved in conjugation of transfer genetic material between bacterial cells. (Bannam et al., 2011). Work carried out on a well characterised antibiotic resistance plasmid, pCW3, containing the same tcp-locus indicates that mutations in genes on this locus can reduce the transfer frequency of plasmids (Porter et al., 2012; Teng et al., 2008). Similar transfer frequencies may be seen with the NetB plasmid as the same mechanisms appear to be present.

To unravel the complex interactions between infectious and predisposing factors to NE a broiler *in situ* model was recently developed (Athanasiadou et al., 2015). These types of models have been used in a variety of species to investigate invasion of host tissue over time (Girard-Misguich et al., 2011; Paulin et al., 2007). Intestinal loop studies have been also used to investigate bacteria colonisation mechanisms in poultry. Loops in layer hens injected with different *Salmonella* serovars have been used to characterise how some invade the gut differently from others (Aabo et al., 2002, 2000). These

studies can be useful in determining which times it may be beneficial to treat a particular condition or disease.

In situ models, like the one recently developed to investigate NE, allow for a controlled exposure of the duodenum to toxin from *C. perfringens* which ensures the quantity administered is the same across all birds, removing any variability induced by transit through the stomach. . With the controlled conditions of the experimental set up there is less environmental variation between birds and the *in situ* model also allows for early responses to be detected. House musk shrew and rabbit intestinal loop models have been used to detect changes after exposure to *Staphylococcal* enterotoxin A. This loop model allowed for gross changes, such as swelling of the intestine to be detected. Positive control loops were swollen but the toxin showed similar results to the negative control (Maina et al., 2012). *In situ* models allow investigation in to the underpinning mechanisms of the disease pathogenesis and the response of the host against the bacteria or the culture supernatant. This also allows for the response to disease causing components to be separated and give clearer indications of how controls may be put in place to prevent disease.

These models also allow for two or three treatments to be tested in one animal, further reducing the number of animals. Recently loops created in rabbit ileum were used to determine effects of *Clostridium difficile* toxin A. Alanyl-glutamine, which improves gastrointestinal muscle structure and function after injury, and an adenosine A_{2A} receptor agonist, which reduces the production of inflammatory cytokines from immune cells, were used as

treatments in some loops. These compounds were found to prevent the damage caused by *C. difficile* toxin A (Warren et al., 2012). Using the *in situ* model allowed for both the toxin and the treatments to be tested within one rabbit along with controls. In a similar manner, temporal data can be obtained by removing tissue biopsies from a loop at different time points to show how host responses and/or pathogen responses change over the duration of the experiment. These types of studies can provide useful insights into how early host responses are initiated within an appropriate tissue. However, to study long-term changes in the immune response in the days and weeks after an infection, we still need a robust broiler infection model.

1.6 Thesis Aims and Main Objectives

As discussed above, host responses to *C. perfringens* are not particularly well characterised and it has been difficult to unravel the response to secreted toxins compared with bacterial cells. In particular, responses have not been investigated in the hours after exposure. Differences in early immune activation may provide insights into *C. perfringens* colonisation of the broiler intestine. The mechanisms behind NE pathogenesis are also not well understood. Therefore, the main aims of this thesis were to:

i) Characterise innate responses to *C. perfringens* and the toxins it produces in culture by
    a) Investigating immune cell populations in the duodenum of broilers via histology and immunohistology and
    b) The expression of genes related to inflammation and immune cell activity via qPCR.

ii) Explore mechanisms for disease susceptibility in broilers by

a) Using isolates which have different virulence profiles (e.g. NetB+ compared with NetB-) and

b) Comparing responses in two commercial breeds to explain disease susceptibility.

This will provide a better understanding of the initial host response and whether this is differentially activated in response to different *C. perfringens* factors. This then may provide an insight as to which responses are beneficial to the broiler for protection against NE.

Chapter 2 : Broiler intestinal responses to *C. perfringens* culture supernatant *in situ*

Adapted from: S. Athanasiadou, KM Russell, P Kaiser, T Kanellos, STG Burgess, MA Mitchell, RE Clutton, SW Naylor, MR Hutchings, NH Sparks. Genome-wide transcriptomic analysis identifies pathways affected by the infusion of *Clostridium perfringens* culture supernatant in the duodenum of broilers in situ. The Journal of Animal Science. 93:6,p3152-3163

2.1 Introduction

Necrotic enteritis is considered to be on the increase as the pressure to reduce antibiotics from governments and consumers rises. In the literature, experimental models are carried out in various ways to reproduce NE to investigate host responses to *Clostridium perfringens*; the causative agent of this disease. Such models take into account different predisposing factors and use *C. perfringens* isolates of variable virulence. A recent review discusses some of the different methods used to induce NE in broilers and the many variables that should be considered when using NE experimental models (Shojadoost et al., 2012). Factors such as the bacterial isolate, diet, co-infection, and the number of doses and the method of administration should be taken into account to reproduce the disease (subclinical or clinical). The variability between models used can hamper the interpretation of results between different studies.

As a consequence of this variance, host responses to NE are still not well characterised, particularly at the early stages of exposure to the bacteria, the toxins and other antigens that they produce. Selecting broilers for innate pro-inflammatory mediators increased their resistance to bacterial infection with *Salmonella enteritidis* (Swaggerty et al., 2014). Investigating innate responses to *C. perfringens* antigens may highlight mechanisms by which protective responses are initiated. Once these responses are determined it could be possible in the future to design disease interventions to promote these protective responses or even select broilers for increased resistance to *C. perfringens*.

In situ models have been used to investigate the pathogenesis of various bacterial isolates and also toxins produced by bacteria. Surgery is performed, loops are created in the intestine which are then infused with the culture preparations of interest (Aabo et al., 2002, 2000; Anvari et al., 2012; Chadfield et al., 2003; Maluta et al., 2014; Warren et al., 2012). A genome wide gene expression study previously carried out using an *in situ* broiler model highlighted a number of pathways significantly differentially regulated in birds infused with *C. perfringens* culture supernatant, including cell growth and proliferation, cell death and cell to cell signalling (Athanasiadou et al., 2011).

The objective of this study was to characterise the effect of *C. perfringens* culture supernatant on the broiler duodenum soon after exposure. We aim to describe temporal responses to *C. perfringens* antigens using an *in situ* loop model. A crude culture supernatant (rather than a purified toxin) was used to capture broiler responses to as many secreted components of *C. perfringens* as possible. Over the last decade, new virulence factors have been discovered in *C. perfringens* in poultry. These include NetB, a pore forming toxin (Keyburn et al., 2008; Savva et al., 2013), and TpeL (Coursodon et al., 2012). It is possible that a number of other virulence factors are yet to be discovered in NE causing isolates of *C. perfringens*.

2.2 Materials and Methods

2.2.1 *In vitro* experiments:

2.2.1.1 Bacterial isolate characterisation: *C. perfringens* type A isolates MPRL 4733 and 4739 which were isolated from clinical cases of NE (SRUC Veterinary Services) were cultured from freeze dried stocks onto sheep blood agar plates and allowed to grow anaerobically at 37°C overnight. PCR characterisation of toxinotyping and virulence genes was carried on isolates for the *in situ* experiment. A single, well isolated colony was removed from the plates and placed in 100µl of sterile RNAse/DNAse free water. This was boiled for 10 minutes, centrifuged at 14000g for 10 minutes and the supernatant was removed. PCR was carried out to test for the presence of *C. perfringens* alpha, beta, epsilon, iota, beta-2, enterotoxin, netB and TpeL toxin genes in each isolate supernatant. Phire Hot Start II DNA polymerase (Finnzymes, Thermo Scientific) was used in 20µl reactions. Previously characterised *C. perfringens* isolates, CP4 (netB positive) and CP5 (netB negative) (from Prescott group, Guelph, Canada (Lepp et al., 2013)) were used as controls where applicable. Primer details are shown in Table 2.1.

Table 2.1. Primers for *C. perfringens* toxinotyping and virulence genes

Gene	Forward Primer	Reverse Primer	Product length
Alpha	GCTAATGTTACTGCCGTTGA	CCTCTGATACATCGTGTAAG	324bp
Beta	GCGAATATGCTGAATCATCTA	GCAGGAACATTAGTATATCTTC	195bp
Epsilon	GCGGTGATATCCATCTATTC	CCACTTACTTGTCCTACTAAC	655bp
Iota	ACTACTCTCAGACAAGACAG	CTTTCCTTCTATTACTATACG	446bp
NetB	GCTGGTGCTGGAATAAATGC	TCGCCATTGAGTAGTTTCCC	384bp
Beta-2	AGATTTTAAATATGATCCTAAC	CCAATACCCTTCACCAAATACTC	567bp
TepL	ATATAGAGTCAAGCAGTGGAG	GGAATACCACTTGATATACCTG	466bp
Enterotoxin	GGAGATGGTTGGATATTAGG	GGACCAGCAGTTGTAGATA	655bp

2.2.1.2 Crude culture supernatant production: (Previously carried out) Two *C. perfringens* Type A isolates (MPRL 4733 and 4739) were cultured overnight in brain heart infusion (BHI) broth (Oxoid Limited, Thermo Fisher Scientific, Hertfordshire, UK), anaerobically. Thioglycolate toxin medium (TTM) (Acumedia, Neogen, Lansing, MI) was inoculated with BHI cultures and incubated anaerobically for 3 hours at 40^0C. The TTM culture was centrifuged and the supernatant precipitated with ammonium sulphate. The precipitate was discarded after centrifugation at 0^0C for 20 minutes. Precipitation and centrifugation was repeated and the supernatant was discarded. The pellet was then resuspended in distilled water. A final centrifugation was carried out to remove any insoluble material. The control

preparation was performed in the same way except bacteria was excluded from the BHI broth.

2.2.1.3 *In vitro* cytotoxicity Assay for NetB: The presence of functional NetB in the culture supernatant was confirmed visually, via microscopy, and quantitatively, using a modified cytotoxicity assay based on one described by Smyth and Martin (2010). The use of a real time cell analyser xCelligence DP system (ACEA Biosciences, Inc) which utilises impedance signals to quantify adherent cell proliferation and viability was used to quantify the cytotoxicity of culture supernatant in real time (Limame et al., 2012). The chicken liver male hepatocyte (LMH) cell line was maintained at 5% CO2 and 37°C in Weymouth's MB 752/1 medium supplemented with 10% foetal calf serum, 1% chicken serum, 100 U/ml penicillin and 100 U/ml streptomycin for the duration of the experiment (Kawaguchi et al., 1987). xCelligence E-plates were coated with gelatin (0.1%, Embryomax solution, Millipore) to allow LMH cells to adhere. 50µl of Weymouth's MB 752/1 medium was added to each well and the plate was inserted into the xCelligence DP system to measure the background impedance. LMH cells (100, 000) were then added to each well in 50µl of medium and the E-plates were returned to the xCelligence. The cells were allowed to settle on the base of the well for 30 minutes prior to cell index readings being taken. Cell index readings were taken every 30 minutes for 24 hours. After this period, the plates were removed and 100ul of a 1:2 dilution series of crude culture was added to the wells in duplicate starting with 1:2 dilution of the neat crude culture preparation. The E-plates were returned to the DP system and cell index

readings continued every 30 minutes for a further 60 hours. Cytotoxicity was associated with a reduction in cell index, as previously described (Huang et al., 2014; Ryder et al., 2010).

2.2.2 *In situ* experiment

2.2.2.1 *C. perfringens* culture supernatant infusion *in situ*: (Previously carried out) 14 three-week old Ross male broilers were housed together to ensure the same rearing conditions and similar intestinal development. Prior to surgery, birds were assigned to receive either *C. perfringens* culture supernatant (n=9) or the control preparation (n=5). Food and water were withheld for 1h prior to anaesthesia which was induced by isoflurane (Isoflo; Abbott Laboratories, Maidenhead, UK) in a purpose-built chamber. Tracheal intubation was performed after anaesthesia had deepened allowing birds to be maintained with aspirated isoflurane preventing motor and autonomic nervous responses to surgery. A 5cm transverse incision was made allowing the duodenum to be identified and withdrawn from the body cavity. Four loops were created in the duodenum using ligatures and either culture supernatant or the control preparation was injected into the lumen. Loops were removed from each bird at 0.5, 1, 2 and 4 hours after infusion. Each loop was split into two and was fixed in either 10% formalin for histology analysis or RNA-later (Sigma-Aldrich, Dorset, UK) for gene expression analysis.

2.2.2.2 Heterophil quantification: Duodenal tissue was fixed in 10% formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin. Slides were examined under x400 magnification for

the quantification of heterophils. Five high powered fields were chosen throughout the section and the number of heterophils counted in each. The mean number for the five fields of view in each slide was calculated.

2.2.2.3 Gene Expression analysis: Gene expression analysis was performed with RT qPCR. The genes targeted for the analysis were related to disease pathogenesis and innate immune responses as identified in previous studies (Athanasiadou et al., 2011). Duodenal tissue was stored at -80°C until RNA extraction. Precellys lysis tubes (Stretton Scientific, Stretton, UK) were used for homogenising tissue with the RNeasy kit (Qiagen) to extract RNA from the tissue (used to manufacturer's specifications). RNA was converted to cDNA using a Verso cDNA kit (Thermo) and was stored at -20^{0}C. Quantitative PCR was carried out using an Mx3000 thermocycler (Stratagene). Brilliant III Ultra-Fast SYBR Green qPCR Mix (Agilent) was used with 1µl of cDNA (diluted 1:10 in nuclease free H2O) in a 20µl reaction. The thermal cycle conditions were 95^{0}C for 3 minutes and then 40 cycles of 95^{0}C for 20 seconds then 20 seconds of a primer specific annealing temperature. Purity of the product was determined using a melting curve. The primer sequences are shown in Table 2.2. Standard PCR conditions were used to obtain the product of each amplicon. PCR products were purified and quantified using a Nanodrop™ spectrophotometer (Thermo Scientific) and used to produce standard curves for the determination of relative concentrations. PCR products were sequenced (Eurofins, Germany) to verify that correct products were amplified. PCR products were diluted to produce top standards which were detectable during qPCR amplification at around

14-16 cycles, with seven ten-fold serial dilutions forming the standard curve (Gong et al., 2010). Expression values were normalised to the reference genes SF3A1 and β-actin. Their geometric mean was calculated and used for normalisation. SF3A1 and B-actin were determined as the most reliable normalisation genes by the Gallus gallus 6 gene geNorm kit (PrimerDesign Ltd) and Qbase plus software.

Table 2.2. Primers for gene expression analysis

Gene name	Forward Primer	Reverse Primer	PCR Size (bp)	Annealing temp (°C)
FAS	CCTGACCCACCACGT CCCTGA	GGTTTCGTAGGCTCCTC CCATTCCA	196	60
KIT	CGGATCCTGGTCGA GAGCACTGT	GCGGCGACCCCAAATGC GATTA	176	61
IRAK4	TGGCAGAAACGTGG CTGTCAAGA	ACCAAACAGGGCTGAGC ACCATC	165	65
B-LA	ACGTCCTCATCTGCT ACGCCGA	TTCCGGCTCCCACATCC TCTGG	236	60
NBL1	CGGCTGCGAGTCCAA GTCCATC	TCCACCAGCTTGTCAAC CCTGG	200	60
GIMAP8	TCGTGGGCAAGACG GGGAGT	CCGCAGAAGCGGCCTTT AGC	130	65
BCL6	CCCCAAGCGAGCAGA CTCAACAAC	AGGCTGAGCCAGAGGT GTGAA	200	60
IL-6	TGTGCAAGAAGTTCA CCGTGT	TTCGTCAGGCATTTCTC CTCGT	130	60
IL-1B	GTGAGGCTCAACATT GCGCTGTA	TGTCCAGGCGGTAGAA GATGAAG	214	65
IFN-γ	ACACTGACAAGTCAA AGCCGC	AGTCGTTCATCGGGAGC TTG	129	65
B-Actin	GAGAAATTGTGCGT GACATCA	CCTGAACCTCTCATTGC CA	180	60
SF3A1	Not disclosed	Not disclosed		60

2.2.3 Statistical analysis

Genstat Version 15 was used for statistical analysis. A repeated measures ANOVA was carried out to compare the treatment effects of the culture supernatant on gene expression results over time. Log10 transformation was carried out on gene expression data to improve the normality of the residuals which were plotted on a histogram and normal plot. Where data sets included zeros, log10+1 transformation was used to improve normality of the data and to ensure all samples were included in the analysis. A one-way ANOVA was also carried out at each time point to compare culture supernatant-treated compared with control. Heterophil data was log10 transformed for the same reasons as above and analysed with a repeated measures ANOVA.

2.3 Results

2.3.1 *In vitro* experiments

2.3.1.1 Bacterial isolate characterisation: Isolates originating from clinical cases of Necrotic enteritis were confirmed as type A by using PCR to confirm the alpha-toxin gene and ensuring the isolates were negative for beta, iota and epsilon toxins. These results are shown in Table 2.3. The isolates were tested for the presence of other virulence toxins. The gene encoding netB, was detected in MPRL4739. MPRL4733 was negative for netB. This was the only isolate from the clinical cases which was found to be positive for this toxin gene. Both isolates from the clinical cases were positive for the β2 gene. Neither of the isolates taken from clinical cases were TpeL positive.

Table 2.3. PCR results for isolates from clinical NE cases

Isolate	Alpha	Beta	Itoa	Epsilon	Beta-2	Enterotoxin	Net-B	TpeL
4733	+	-	-	-	+	-	-	-
4739	+	-	-	-	+	-	+	-

The table summarises the PCR results for toxin genes present in *C. perfringens* isolates taken from cases of NE. + indicated when a positive band was detected. – indicates a negative result for the gene.

2.3.1.2 Cytotoxicity Assay for NetB: LMH cells cultured to 80% confluency in a standard 24 well cell culture plate were incubated with *C. perfringens* culture supernatant in a two-fold dilution sequence across the plate for 16 hours at 37° and 5% CO2. Cells were observed at x100 magnification. Culture supernatant from *C. perfringens* (isolates 4733 and 4739) caused cytotoxicity (rounding and detachment of cells) at a dilution of 1:16 visible by microscopy. This culture supernatant was considered to be positive for NetB. Figure 2.1 shows images of cells with Weymouth's medium (A), a toxin free preparation (B), culture supernatant 1:2 (C) and culture supernatant 1:16 (D).

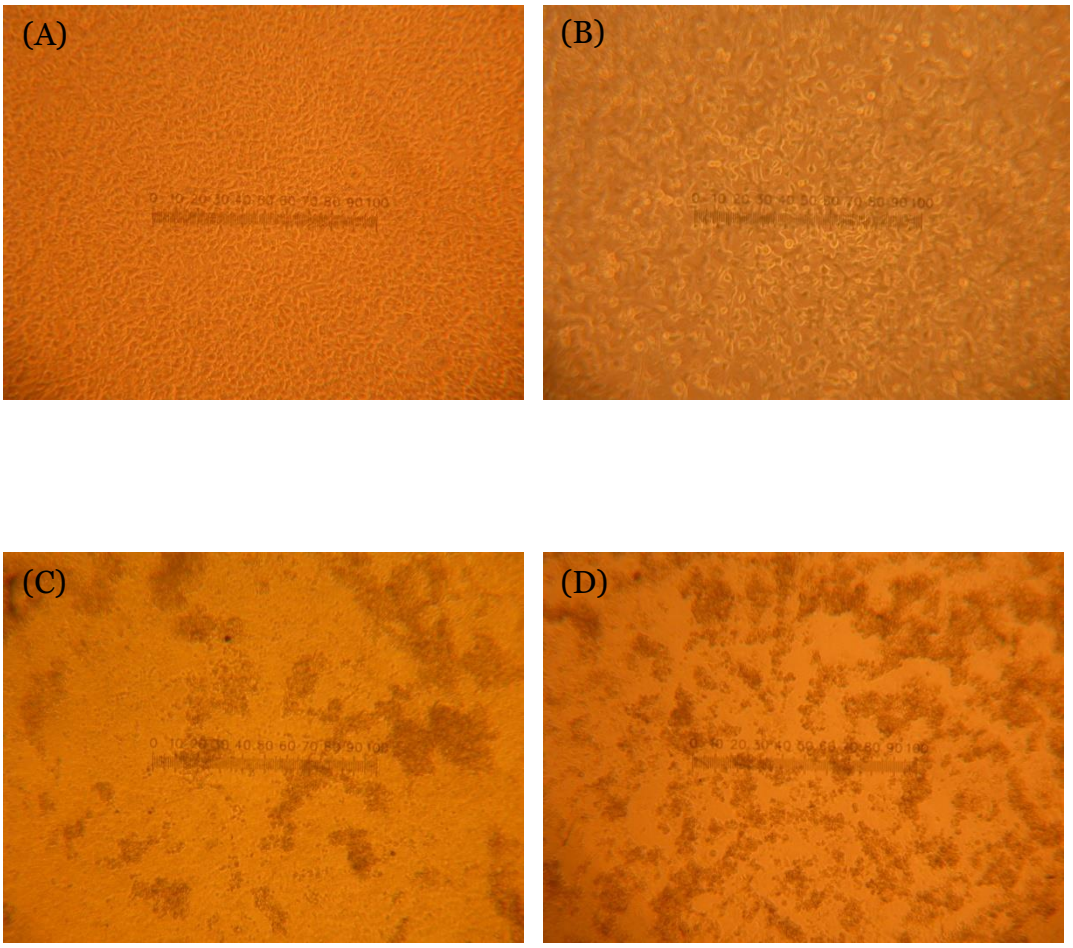

Figure 2.1. Cytotoxicity assay for NetB (microscopy)

(A) Cells incubated with Weymouth's MB 752/1 medium only. (B) Cells incubated in toxin free control preparation (Thioglycollate toxin medium) used as control for duodenal loop experiment. (C) *C. perfringens* culture supernatant (1:2 dilution). (D) *C. perfringens* culture supernatant (1:16 dilution). (C) and (D) show cell rounding and detachment from the well.

The RTCA xCelligence DP system was used to quantify the cytotoxicity of NetB+ culture supernatant (Figure 2.2). The quickest reduction in cell index was evident in wells incubated with the lowest dilution of culture supernatant (1:2). LMH cells incubated with this level of culture supernatant die soon after its addition. This effect diminishes as the culture supernatant is diluted further. The 1:16 dilution is cytotoxic to LMH cells indicating that this supernatant is NetB positive.

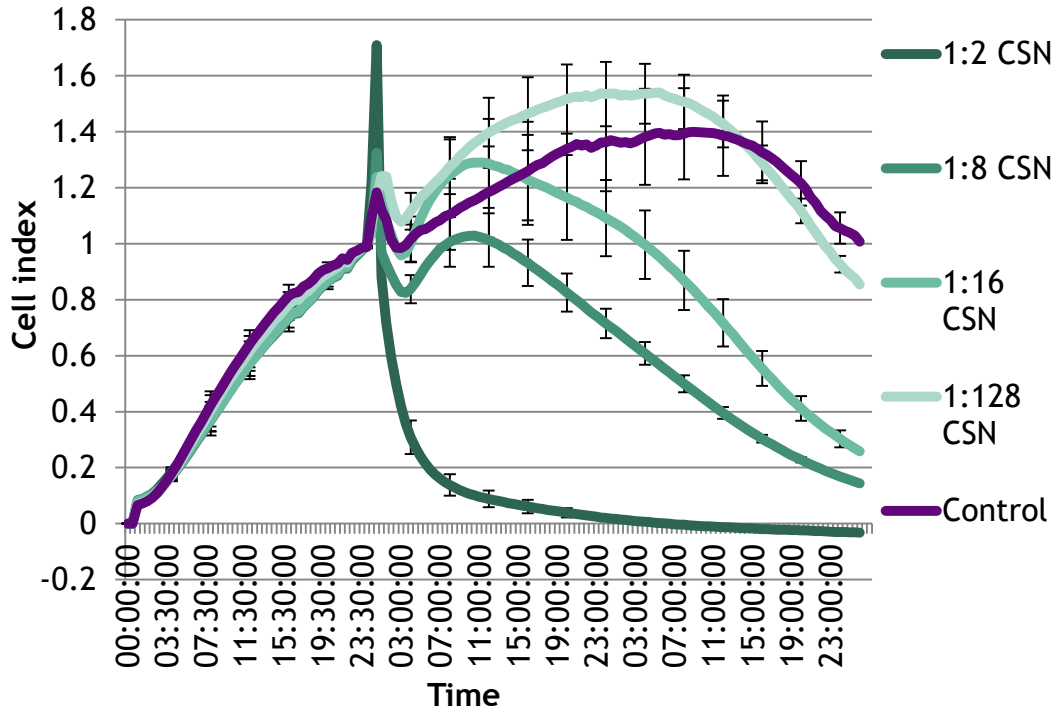

Figure 2.2. Cytotoxicity assay for NetB (RTCA)

Concentration and time dependant effects of *C. perfringens* strain MPRL 4733 and 4739 culture supernatant on LMH cells using the RTCA xCelligence system. Each concentration of culture supernatant was run in duplicate and curves show the average cell index over three assays ±SE. CSN= Culture supernatant.

2.3.2 *In situ* experiment

2.3.2.1 Heterophil Quantification: Heterophils were detected in H&E stained sections at all time points in control and culture supernatant infused birds. Results showed an increase in heterophil numbers over time ($p<0.001$) in both treatment groups but no effect of the culture supernatant was detected over the time period investigated, indicating that these *C. perfringens* antigens did not attract heterophils locally ($p=0.73$) (Figure 2.3).

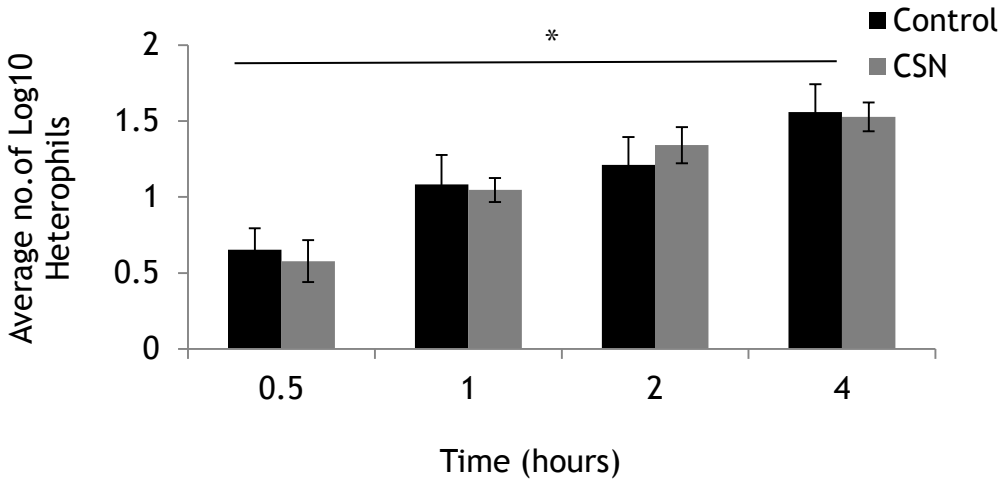

Figure 2.3. Heterophil quantification after culture supernatant infusion
Heterophil quantification over four hours of culture supernatant exposure. Heterophils were counted in five high powered fields for each bird. Averages with SE are shown. Control n=5, Culture supernatant n=9. CSN= Culture supernatant. * indicates significant increase in heterophils over time.

2.3.2.2 Analysis of normalisation genes: ANOVA analysis was performed on the geometric mean of the normalisation genes. This indicated no difference in mRNA expression between the control and CSN treatments

(p=0.08). There was a significant increase of the geometric mean after the 0.5h time point (p=0.002) as indicated in Figure 2.1.

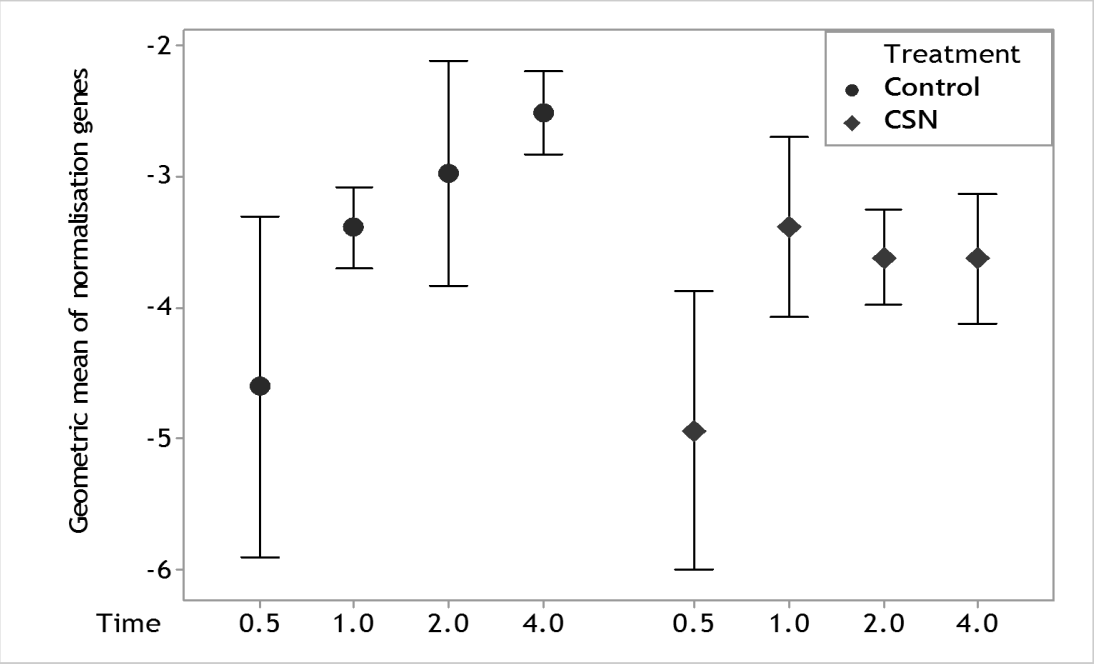

Figure 2.4. Changes in normalisation mRNA expression

Log10 transformed geometric mean of the two normalisation genes used during qPCR analysis. The normalisation genes increase after the initial 0.5h time point then remain constant across the experiment. No difference was detected between the control and culture supernatant treatments.

2.3.2.3 Gene Expression Analysis: A number of genes were measured in this study to determine the effect of culture supernatant over the first four hours of exposure. The genes were selected based on transcriptional analysis that identified their involvement in biological processes and pathways one hour post exposure to culture supernatant (Athanasiadou et al., 2015). These processes were i) cell morphology and death (cell death receptor, *FAS*, GTPase IMAP family member 8 [*GIMAP8*], vitronectin [*VTN*]), ii)

inflammatory responses (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene [*KIT*], interferon γ [*IFN-γ*], interleukin 6 [*IL-6*] and interleukin 1-β [*IL-1β*]), iii) immune cell trafficking (interleukin 1 receptor associated kinase 4 [*IRAK4*], neuroblastoma 1 [*NBL1*] and B cell Lymphoma 6 [*BCL6*], and iiii) antigen presentation (MHC class II α chain [*B-LA*]). The results are presented in Table 2.4. Figure 2.4 includes key results discussed in section 2.4 of this chapter.

There were no significant interactions with the loop treatments over time, however, *FAS* and *NBL1* both showed a tendency to be increased in culture supernatant treated birds in comparison to the control birds (p=0.06 and p=0.085). This increase was most evident at the 2h time point for both genes.

Two genes involved in immune cell trafficking were affected by the loop treatment. A significant increase was detected in *BCL6* mRNA expression levels in culture supernatant treated birds compared with the control birds (P=0.043). *NBL1* mRNA expression tended to be increased in toxin treated broilers (P=0.059). The mRNA expression of the inflammatory response genes, *IFN-γ* and *IL-6*, also tended to be higher in culture supernatant infused broilers when compared with the control (P= 0.081 and 0.087 respectively). None of the other genes related to innate inflammatory responses (e.g. *IL-1β, B-LA*) or those related to cell morphology and death (e.g. *FAS, GIMAP8, VTN*) were found to be affected by the culture supernatant.

There were significant changes in the mRNA expression levels over the 4 hours of the majority of the genes measured here. Inflammatory cytokine

expression differed across the time points investigated as *IL-6* ($p<0.001$) increased between 0.5h and 2h but then diminished at 4h while *IFN-γ* ($p=0.018$) mRNA expression was highest at 0.5h and decreased until the final 4h time point. Four of the other genes measured (*KIT, BCL6, B-LA, VTN*) had similar expression profiles to that of *IFN-γ* ($p=0.023$, $p<0.001$, $p<0.001$, $p<0.001$). Expression levels of these genes peaked at 0.5h and reduced thereafter. *FAS* and *NBL1* mRNA expression showed a similar pattern to these genes initially but expression levels increased again at 4h. *IL-1β*, *GIMAP8* and *IRAK-4* mRNA was similarly expressed at all four time points analysed here.

Table 2.4. Values are the mean log transformed values for both control and culture supernatant infused broilers. The standard error of the deviation and the P values are presented from the repeated measures ANOVA for loop treatment, time and the interaction between the two. (Table to follow on p53).

Table 2.4. Mean transformed gene expression analysis results for control (CTRL) and Culture supernatant (SN) over four hours

Data trans-formation	Gene	0.5h		1h		2h		4h		Loop treatment		Time		Time.Loop treatment	
		CTRL	Culture SN	CTRL	Culture SN	CTRL	Culture SN	CTRL	Culture SN	SED	P	SED	P	SED	P
Log+1	*IFN-γ*	2.44	2.07	1.35	1.8	1.1	2.18	0.58	1.17	0.229	0.081	0.334	0.018	0.485	0.249
Log+1	*IL-6*	0.296	0.465	0.385	0.503	0.491	1.054	0.002	0.003	0.1143	0.087	0.1319	<.001	0.2037	0.233
Log+1	*IL-1β*	0.096	0.042	0.055	0.061	0.002	0.114	0.013	0.033	0.026	0.432	0.038	0.496	0.0551	0.249
Log+1	*BLA*	0.753	0.619	0.394	0.726	0.027	0.081	0.001	0.014	0.091	0.479	0.1339	<.001	0.1938	0.376
Log+1	*IRAK-4*	1.90E-04	1.60E-04	1.00E-05	1.00E-05	8.70E-04	3.64E-03	1.00E-05	0.00E+00	1.13E-03	0.557	1.50E-03	0.245	2.22E-03	0.542
Log+1	*KIT*	0.0143	0.0287	0.0004	0.0007	0.0015	0.0099	0.0031	0.0007	0.00347	0.161	0.00644	0.023	0.00893	0.484
Log+1	*GIMAP8*	2.61	2.22	1.86	2.07	1.93	2.35	1.8	2.62	0.327	0.432	0.32	0.571	0.523	0.337
Log	*FAS*	7.892	8.079	6.732	6.668	5.52	6.242	7.213	6.992	0.1175	0.211	0.1505	<.001	0.2254	0.066
Log	*BCL6*	4.573	5.169	3.648	3.843	2.895	3.516	3.737	3.877	0.1711	0.043	0.2591	<.001	0.3728	0.604
Log	*NBL*	3.827	3.643	2.869	3.075	1.421	2.356	2.562	2.828	0.1465	0.059	0.1819	<.001	0.2747	0.085
Log	*VTN*	1.456	1.479	0.746	0.532	-0.497	-0.021	0.322	-0.025	0.1542	0.92	0.2135	<.001	0.3135	0.283

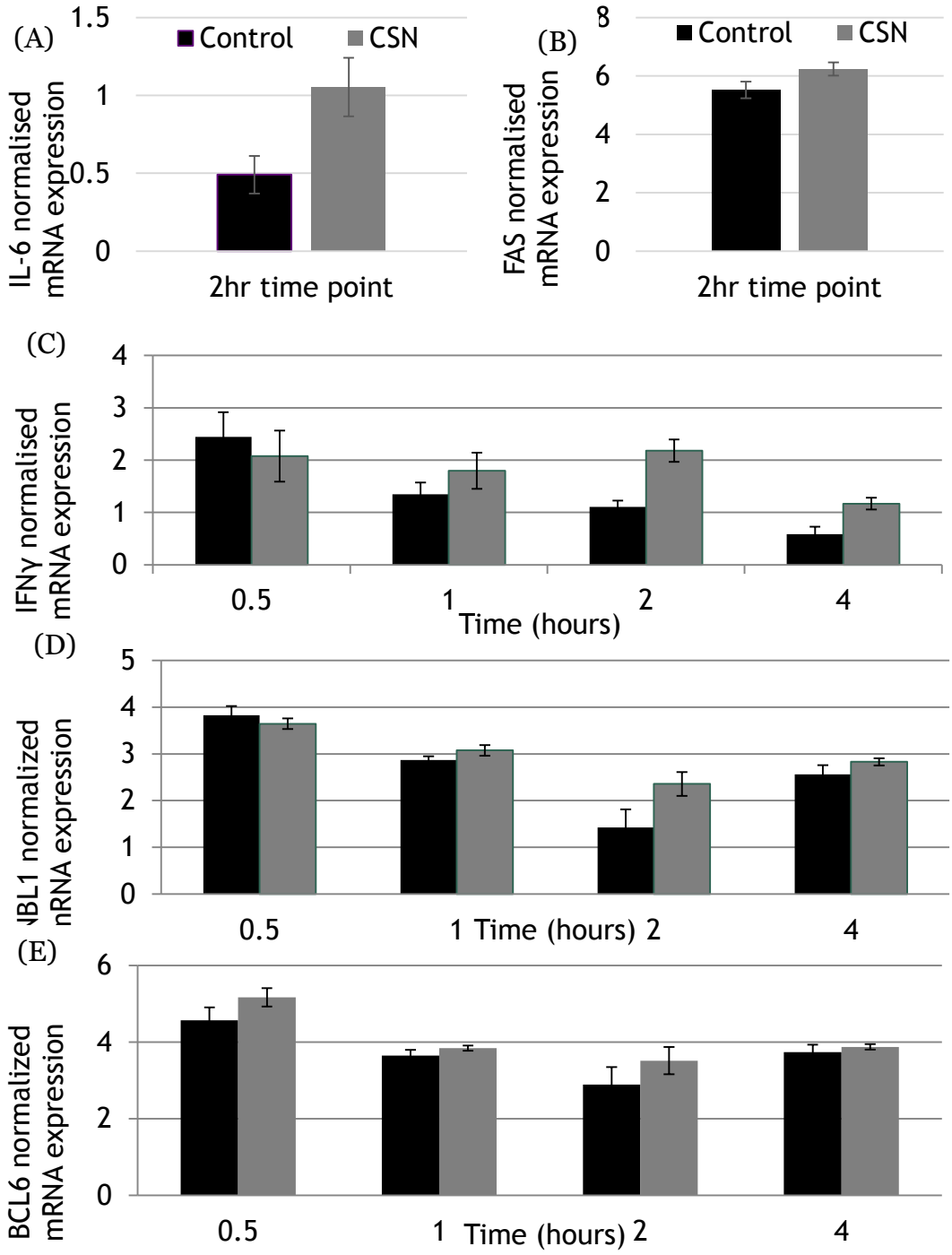

Figure 2.5. mRNA expression for selected genes

mRNA expression data for selected genes referred to further in the discussion. A) and B) present the 2 hour data for IL-6 and FAS respectably where both genes tended to be increased in the culture supernatant treated

birds. C), D) and E) present data at all four time points included in the experiment for IFNγ, NBL1 and BCL6.

2.4 Discussion

In this experiment we characterised isolates of *C. perfringens* originating from clinical cases of NE and used culture supernatant from them in an *in situ* loop model identify early avian responses to components from *C. perfringens*. We have detected early changes in the expression of genes related to immune cell activity and cell viability as well as moderate differences in pro-inflammatory cytokine levels after exposure to culture supernatant.

PCR confirmed the genetic characterisation of the two isolates that derived from clinical cases of NE. Of these two, only one was found to possess the netB virulence gene. Previous studies indicated that not all isolates from clinical cases of NE had the netB gene but they were more likely to possess it than *C. perfringens* isolated from healthy birds (Johansson et al., 2010; Keyburn et al., 2010). These isolates were used to produce the culture supernatant, which was then infused into the duodenal loops of birds during the *in situ* experiment. We have confirmed the presence of NetB in the supernatant by qualitative and quantitative methods.

Qualitatively, the presence of NetB was determined by the observation of cytotoxicity against LMH cells, at dilution greater than 1:8 (Keyburn et al., 2008). Cells were rounded and no longer adherent to the well when incubated with culture supernatant diluted 1:16 for 16 hours (Figure 2.1.). Here, we also used a real time cell analyser to quantify the activity of *C.*

perfringens culture supernatant on LMH cell growth. To our knowledge this is the first time impedance-based technology has been used to observe the actions of *C. perfringens* culture supernatant on an avian cell line. At a dilution of 1:2 the majority of cells were dead in four hours unlike control cells incubated with cell culture media or toxin free control preparation (Figure 2.2). The culture supernatant from isolates MPRL 4133 and 4139 is considered to be NetB positive as it is cytotoxic to LMH cells at the dilution 1:16 (Keyburn et al., 2008; Smyth and Martin, 2010). At dilution 1:16 the cytotoxic effects were not as immediate as the more concentrated culture supernatant but a significant reduction in growth was observed by 24 hours post exposure.

Heterophils are the main polymorphonuclear leukocytes in poultry and act as the first line of defence against microorganisms (Kogut et al., 2001). Increased numbers of heterophils were detected in the duodenum one day post experimental challenge with a NetB negative strain of *C. perfringens* and this was reduced if broilers were heat stressed (Calefi et al., 2014). Larger numbers of heterophils have also been detected in the blood after 3 days of *C. perfringens* challenge (Saleh et al., 2011). Our results showed that heterophil numbers were significantly increased over the four hours of the experimental procedure but there was no significant effect of the culture supernatant treatment. Heterophils also respond to sites of injury to phagocytose cell debris. It is possible in this case that heterophils responded to injury caused by the ligatures applied to create the loops within the duodenum. Heterophils express increased levels of IL-6 and IL-1β mRNA after exposure to toll-like

receptor ligands (Kogut et al., 2006). Although the numbers these cells increased we did not, detect a significant increase in the expression of pro-inflammatory cytokines over time, to match cell increase. A tendency for *IL-6* expression to be increased at 2h in loops infused with culture supernatant may, in part, be related to heterophils present in these loops but *IL-6* is also expressed in epithelial cells, dendritic cells and macrophages. Further work is required to determine what cell types are involved in protective responses to NE and whether the disease can be exacerbated by hypersensitive inflammatory responses. Identifying cells important for protection and the mediators they produce could influence future vaccine development and other disease interventions.

IFN-γ mRNA expression was increased in culture supernatant infused broilers. Broilers co-infected with *Eimeria maxima* and *C. perfringens* had a reduced IFN-γ mRNA expression within the intra-epithelial lymphocyte population in comparison to this single infection (Park et al., 2008). Other experimental co-infections have found an increase in *IFN-γ* mRNA in the ileum in the first few days of the model (Collier et al., 2008). The differences in the levels of *IFN-γ* expression between these studies may be related to the timings of the co-infections in each challenge model used and the different cell populations used for mRNA expression analysis. Regardless of co-infection, *C. perfringens* toxins are likely to be present in the intestine but it is unclear which cells may be responding to these secreted components. *IFN-γ* in our *in situ* model may be produced by NK-cells and γδ T-cells residing in the lamina propria and intra-epithelial layer. In mammals, NK cells form part

of the innate lymphoid cell (ILC) population in mammals along with ICL1 cells. ICL1 cells also produce *IFN-γ* but their functions in homeostasis and disease are currently not well characterised (Crellin et al., 2010; Walker et al., 2013). It is unknown whether these subsets of ILCs cells exist in the chicken and if they could play a role in protection against intestinal pathogens. Further work is required to determine which secreted components from *C. perfringens* are recognised to induce the up-regulation of IFN-γ, which cells are contributing to this response and if this promotes clearance of NE.

Cell death is a histological feature of NE in birds (Olkowski et al., 2008, 2006). There are currently thought to be various processes which mediate cell death in a variety of ways. Some of these are apoptosis, necrosis and necroptosis. Apoptosis and necroptosis are both regulated forms of cell death but necrosis is not programmed and occurs when cells undergo too much stress (Gunther et al., 2013). Although it is not fully understood which mechanism is the predominant one that mediates cell death during NE, it appears possible that a balance occurs between them all. Increased apoptotic cell death occurs in the intestine of broilers during NE compared with uninfected control birds (Liu et al., 2012). Alternatively, genes involved in apoptotic cell death, such as FAS, FLIP, Caspase 8 and Caspase 9, are down-regulated in the spleen in the days after *C. perfringens* challenge (Zhou et al., 2009). The reduced expression of these genes in the spleen may be a protective response to boost cell numbers which can act towards the intestinal infection and apoptotic cells from the intestine (Bronte and Pittet, 2013). In our *in situ* experiment, expression of the death receptor, *FAS*,

tended to be increased in culture supernatant treated birds at two hours post infusion. The FAS receptor can initiate apoptosis and necroptosis when it is bound with its ligand on the cell surface (Vandenabeele et al., 2010). Necrotic cell death is noted in the intestine of birds with NE (Olkowski et al., 2006). Necroptotic cells have a similar microscopic morphology to those which have undergone necrosis. This mechanism has still to be shown in the chicken but could be initiated to induce immune mediators at the site of infection (Pasparakis and Vandenabeele, 2015). This study provides a snap shot of disease pathogenesis in the early hours after culture supernatant infusion but there is still much to be understood. For example, it is unclear whether cell death is induced by the bacteria or is mediated by the host. Cell debris initiated from regulated and un-regulated cell death pathways induce different inflammatory pathways. Interventions in the mechanisms of cell death could inhibit the formation of NE lesions in the future.

Culture supernatant infusion caused the up-regulation of *BCL6* throughout the experiment. *BCL6* is required for B-cell development in the germinal centre. This gene is known as a transcriptional repressor (Basso and Dalla-Favera, 2012; Okada et al., 2012). It has also been associated with macrophage responses to infection. Bone marrow derived macrophages derived from *BCL6* knockout mice had increased IL-18 responses after stimulation with LPS in comparison to cells from wild type mice (Takeda et al., 2003). *BCL6* may prevent a hyper responsiveness to bacterial antigens as inflammatory cytokines and chemokines are down regulated by *BCL6* activation (Toney et al., 2000; Yu et al., 2005). *NBL1* also tended to be

increased in culture supernatant infused broilers. Up-regulation of this gene is associated with the prevention of monocyte chemotaxis (Chen et al., 2004). We do not know if the toxin actively up-regulates *BCL6* and *NBL1* or whether the bird activates these genes to prevent damage caused by inflammation. Further *in vitro* investigation into macrophage and other innate cell activities in the presence of *C. perfringens* culture supernatant could clarify whether toxins produced have a direct effect on these cells. Testing individual purified *C. perfringens* toxins as well as the culture supernatant containing a variety of virulence factors may elucidate the role each virulence factor plays during NE infections.

Although further investigation is required this study has provided some insights into the early responses to *C. perfringens* culture supernatant. *In vitro*, toxicity to LMH cells occurred soon after exposure to *C. perfringens* culture supernatant but this effect was alleviated by diluting the supernatant. We expected to detect changes in cell viability in intestinal cells exposed to culture supernatant in the intestinal loop model and our findings indicate that various cell death pathways may be activated in the first four hours post exposure with increased FAS mRNA expression. We detect modest changes in the expression of pro-inflammatory cytokines, IFNγ and IL-6, throughout the duration of the experiment. Increased levels observed at 2h in culture supernatant infused broilers are diminished at 4h. Future studies comparing host responses to bacteria as well as culture supernatant may provide insights as to how these components are recognised and which responses they initiate. We also have some evidence from this experiment that macrophage

activity may be altered in the presence of culture supernatant with the increased expression of BCL6 mRNA. This could be defence mechanisms of the host to instigate protective responses or a virulence property of *C. perfringens* culture supernatant to promote beneficial growth conditions, evade host immunity and cause persistent infection. Further work is required to determine whether this is a host mediated response or not.

This study detected modest changes in pro-inflammatory responses after toxin infusion. In the next chapter the host response to culture supernatant with and without bacteria will be investigated to determine whether the bacterial cells increase the pro-inflammatory response.

Chapter 3 Broiler responses to virulent and avirulent *Clostridium perfringens in situ*

3.1 Introduction

Necrotic enteritis in broilers is caused by *Clostridium perfringens* type A. *C. perfringens* produce a number of antigens associated with disease pathogenesis, including toxins, etc. (Keyburn et al., 2010; Kulkarni et al., 2006; Lanckriet et al., 2010). These contribute to the development of lesions in the duodenum and jejunum of broilers preventing the adequate digestion of food resulting in productivity losses for the industry.

Recent evidence has indicated that one of the key virulence factor for NE in broilers is the presence of NetB toxin (Keyburn et al., 2008). The gene encoding NetB is found on a plasmid which contains a pathogenicity locus (Lepp et al., 2010). When the toxin is produced, seven sub-units of NetB oligomerize in the surface of a cell to create a pore (Savva et al., 2013). In addition to NetB, alpha-toxin, which has been previously considered to be the main virulence factor for NE in broilers, is still believed to play a role in inducing the disease. It is produced by all type A isolates of *C. perfringens* and hydrolyses phospholipids on the cell membrane. Other virulence genes include the TpeL and Beta-2 toxins and Perfrin, a new bacteriocin (Chalmers et al., 2008; Coursodon et al., 2012; Nagahama et al., 2011; Timbermont et al., 2014).

Early broiler responses to virulent *C. perfringens* isolates, i.e. those that produce NetB and other immunogenic proteins associated with NE and avirulent isolates have been poorly described. NetB toxoids and vaccine candidates from virulent isolates tend to infer greater protection against intestinal lesions when compared with avirulent isolates but few studies have

directly compared this (Fernandes da Costa et al., 2013; Kulkarni et al., 2010; Lanckriet et al., 2010). Greater understanding of responses to isolates differing in their virulence may highlight novel vaccine candidates or other routes for disease intervention. Here we aim to investigate early host responses to a virulent isolate and an avirulent isolate in the presence and absence of bacterial cells.

To achieve this we have used the same *in situ* intestinal loop model used in Chapter 2 which allows for a number of treatments to be applied in one bird and for samples to be taken over time (Athanasiadou et al., 2015; Russell Chapter 2). We used this model previously to study host responses to *C. perfringens* culture supernatant in Chapter 2. Here, using this model we tested two hypotheses: i) broiler responses to virulent and avirulent isolates of *C. perfringens* would differ and ii) responses would also be differentially regulated in the presence or absence of bacterial cells.

3.2 Materials and Methods

3.2.1 *In vitro* assays

3.2.1.1 Bacterial isolate characterisation: The presence of genes that encode *C. perfringens* toxins, including beta, epsilon, iota, beta-2, enterotoxin, netB and TpeL was investigated by standard PCR on the two *C. perfringens* isolates that were used in this experiment. Previous studies revealed that isolate CP5 was negative for the netB gene, whereas isolate CP4 was netB positive (Thompson et al., 2006). The isolates were cultured from glycerol stocks on sheep blood agar plates overnight at 37°C anaerobically.

One colony from each plate was transferred to 100µl of RNase/DNase free water. This was boiled for 10 minutes and then centrifuged at 14000g for 10 minutes prior to DNA extraction. The supernatant was removed and used as the sample for each isolate. Phire hot start II DNA polymerase (Finnzymes, Thermo Scientific) was used in 20µl reactions. Primer sequences for the isolate typing and virulence genes are shown in Table 3.1.

Table 3.1. Primers for *C. perfringens* toxinotyping and virulence genes

Gene	Forward Primer	Reverse Primer
alpha	GCTAATGTTACTGCCGTTGA	CCTCTGATACATCGTGTAAG
beta	GCGAATATGCTGAATCATCTA	GCAGGAACATTAGTATATCTTC
epsilon	GCGGTGATATCCATCTATTC	CCACTTACTTGTCCTACTAAC
iota	ACTACTCTCAGACAAGACAG	CTTTCCTTCTATTACTATACG
netB	GCTGGTGCTGGAATAAATGC	TCGCCATTGAGTAGTTTCCC
beta-2	AGATTTTAAATATGATCCTAAC	CCAATACCCTTCACCAAATACTC
tepL	ATATAGAGTCAAGCAGTGGAG	GGAATACCACTTGATATACCTG
enterotoxin	GGAGATGGTTGGATATTAGG	GGACCAGCAGTTGTAGATA

3.2.1.2 Culture of *C. perfringens* for use *in vitro* and *in situ*: Every other day, five to seven bacterial colonies from blood agar plates for both CP4 and CP5 isolates were transferred to 5ml of TPG broth (5% tryptone, 0.5% protease peptone, 0.4% glucose, 0.1% thioglycolic acid) and kept at 37°C anaerobically overnight. This culture was maintained anaerobically at room temperature until use. The evening before surgery, 0.2ml of the culture was transferred to 10ml of TPG broth and kept overnight at 37°C in an anaerobic

jar. Following the incubation, the cultures were centrifuged at 14000g for 10 minutes. The supernatant was removed and kept for further use. The bacterial pellet was re-suspended in 1ml of the culture supernatant (CSN) and was subsequently used in the *in situ* experiment as treatment bacteria + culture supernatant. The bacterial culture used for infusion was approximately 1.5 x10^9 CFU/ml. Aliquots of the culture supernatant were also used in the *in situ* experiment. Excess supernatant from each isolate was stored at -20°C to be used for *in vitro* experiments.

3.2.1.3 *In vitro* cytotoxicity Assay for NetB: As previously described in Chapter 2 Section 2.1.3, Chicken liver male hepatocyte (LMH) cells (ATCC lot number 1878490; Middlesex, UK) were maintained at 5% CO_2 and 37°C in Weymouth's MB 752/1 medium supplemented with 10% foetal calf serum, 1% chicken serum, 100 U/ml penicillin and 100 U/ml streptomycin for the duration of the experiment (Kawaguchi et al., 1987). xCelligence E-plates were coated with gelatin (0.1%, Embryomax solution, Millipore) to allow LMH cells to adhere. 50µl Weymouth's MB 752/1 medium was added to each well and the background impedance was measured by the xCelligence system. LMH cells (100, 000) were then added to each well in 50µl of medium and the E-plates were returned to the xCelligence. The cells were allowed to settle for 30 minutes prior to cell index readings being taken. Cell index readings were taken every 30 minutes for 24 hours. After this initial growth period the plates were removed and 100µl of a 1:5 or 1:25 dilution of either CP4 or CP5 culture supernatant was added to the wells in triplicate. The E-plates were

returned to the xCellignece and cell index readings were taken every 30 minutes for a further 60 hours.

3.2.2 *In situ* experiment

3.2.2.1 *C. perfringens* challenge using an *in situ* duodenal loop model:

Twenty, 18-days old male Ross broilers were housed together to ensure the same rearing conditions and similar intestinal development were used in the experiment. Hatch dates were staggered so that birds were all the same age at the time of surgery. The surgical protocol was previously described by Athanasiadou et al., 2015. Briefly, food and water were withheld for 1h prior to anaesthesia which was induced by isoflurane (Isoflo; Abbott Laboratories, Maidenhead, UK). Tracheal intubation was performed after anaesthesia had deepened allowing birds to be maintained with aspirated isoflurane preventing motor and autonomic nervous responses to surgery. A 5cm transverse incision was made allowing the duodenum to be identified and withdrawn from the body cavity. Ligatures were placed around the duodenum to create six isolated loops. Each of the chambers were assigned to one of the following treatments: control (sterile TPG broth), culture supernatant alone (CSN) or 1.5 x10^9 bacteria + culture supernatant (B+CSN). One intestinal chamber from each treatment was removed at 0.5h post infusion, whereas the other was removed at 4h after treatment infusion. The segment was split into two parts. One part was fixed in 10% formalin for histological analysis or stored in RNA-later for gene expression analysis.

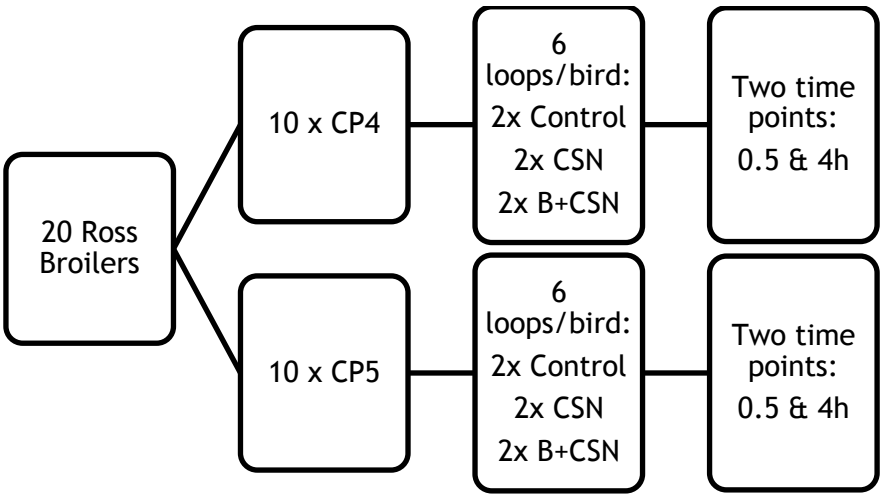

Figure 3.1. Duodenal loop experimental design

Design utilising 20 birds with 10 allocated to isolate CP4 or CP5. Each bird has six loops in the duodenum with one from each of the three treatments being removed at either 0.5 or 4h post infusion.

3.2.2.2 Histological examination: Tissue was fixed in 10% formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E). Sections were scored for pathological lesions in a scale from 1 to 3; Score 1 indicated that there was no pathology change from tissue which had not undergone surgery. Score 2 indicated villi fusion in areas of the section and cell death down to the crypts but no crypt loss. Score 3 indicated villi fusion throughout the section and areas of complete crypt loss.

3.2.2.3 Heterophil quantification: The H&E sections used in the histological examination were examined under x400 magnification for the quantification of heterophils. Five high powered fields were chosen throughout the section and the number of heterophils counted in each, to

calculate the average number of heterophils across these fields of view (Beard et al., 2000). Heterophil quantification was carried out for all duodenal loops, in all birds.

3.2.2.4 Gene Expression analysis: Gene expression analysis was performed with RT qPCR. The genes targeted for the analysis were related to disease pathogenesis and innate immune responses as identified in previous studies (Athanasiadou et al., 2015).

Duodenal tissue from each segment was placed in RNA-later and stored at -80^{0}C until RNA extraction. Precellys lysis tubes (Stretton Scientific, Stretton, UK) were used for homogenising tissue with the RNeasy kit (Qiagen) to extract RNA from the tissue (used to manufacturer's specifications). RNA was converted to cDNA using a Verso cDNA kit (Thermo Scientific) and was stored at -20^{0}C. Quantitative PCR was carried out using an Mx3000 thermocycler (Stratagene). Brilliant III Ultra-Fast SYBR Green qPCR Mix or Brilliant III Ultra-Fast qPCR Mix (Agilent) was used with 1µl of cDNA (diluted 1:10 in nuclease free H_2O) in a 20µl reaction. The thermal cycle was 95^{0}C for 3 minutes and then 40 cycles of 95^{0}C for 20 seconds then 20 seconds of a primer specific annealing temperature which is shown in Table 3.2. Purity of the PCR product was determined using a melting curve. PCR products were obtained for each gene using standard PCR conditions. PCR products were purified and quantified using a Nanodrop™ spectrophotometer (Thermo Scientific) and used to produce standard curves for the determination of relative concentrations. PCR products were sequenced (Eurofins) to verify that correct products were amplified. PCR

products were diluted to produce top standards which were detectable during qPCR amplification at around 14-16 cycles, with seven ten-fold serial dilutions forming the standard curve (Gong et al., 2010).

Expression values were normalised to the geometric means of two reference genes, YWHAZ and SF3A1. These genes were selected from a panel of genes tested from the *Gallus gallus* 6 gene geNorm kit (PrimerDesign Ltd) as the least variable by genorm analysis in Qbase plus software.

Table 3.2. Primer sequences used for qPCR

RNA target	Sequence		PCR size	Annealing temp (°C)
FAS	For	CCTGACCCACCACGTCCCTGA	196	60
	Rev	GGTTTCGTAGGCTCCTC		
IRAK4	For	TGGCAGAAACGTGGCTGTCAAGA	165	65
	Rev	ACCAAACAGGGCTGAGCACCATC		
BLA	For	ACGTCCTCATCTGCTACGCCGA	236	60
	Rev	TTCCGGCTCCCACATCCTCTGG		
NBL1	For	CGGCTGCGAGTCCAAGTCCATC	200	60
	Rev	TCCACCAGCTTGTCAACCCTGG		
GIMAP8	For	TCGTGGGCAAGACGGGGAGT	130	65
	Rev	CCGCAGAAGCGGCCTTTAGC		
BCL6	For	CCCCAAGCGAGCAGACTCAACAAC	200	60
	Rev	AGGCTGAGCCAGAGGTGTGAA		
IL-6	For	TGTGCAAGAAGTTCACCGTGT	130	60
	Rev	TTCGTCAGGCATTTCTCCTCGT		
IL-1β	For	GTGAGGCTCAACATTGCGCTGTA	214	65
	Rev	TGTCCAGGCGGTAGAAGATGAAG		
IFN-γ	For	ACACTGACAAGTCAAAGCCGC	129	65
	Rev	AGTCGTTCATCGGGAGCTTG		
MUC2	For	GCAGCCTTATCCTGAGTGAAATC	88	60
	Rev	CAGGCATCATGAACACAAGCA		
	Probe	TTTGTCATTCCAAGGTGAACCCATCTCC		
MUC5AC	For	CTGAATGTCACTCAACAGTGAA	78	60
	Rev	CAGTGTTCTCACAGTTACATGT		
	Probe	CAGAAGTTTACCAGAAGAACTGCATGTTTG		
MUC13	For	GCAGTAAGGACAGCAGCAGAA	88	60
	Rev	TTGCATCATCACATGGATAAACA		
	Probe	TGCGTCAGTACGCAAATACATCCCAATGT		

Primers for YWHAZ and SF3A1 were purchased from Primer Design Ltd and are proprietary and not disclosed as part of the Gallus gallus genorm kit.

3.2.3. Statistical analysis

Linear mixed-effect models were performed to analyse gene expression and heterophil data using lme from the 'nlme' package (v 3.1-120) in the statistical programme R (v 3.1.2 (c) 2014 The R Foundation for Statistical Computing). The experiment was set up as a 2x3x2 factorial where the isolate (CP4, CP5), loop treatments (control, culture supernatant, bacteria + culture supernatant) and time points (0.5, 4h) were entered into the model as fixed effects; the bird I.D. was included as a random effect to account for the non-independence between loop treatments and time points from the same bird. Initially a maximal model was run with all pair-wise and 3-way interactions included. Step-wise deletion of non-statistically significant terms was then performed sequentially, with the end result a minimal model for the data of only statistically significant interactions and associated main effects. $P<0.05$ was taken to indicate statistical significance throughout. Data from the heterophil quantification and mRNA expression analysis were transformed (Log10 or Log10+1) to normalise the variance before analysis in R. Log10+1 transformations were used when zeros were included in the data set to ensure these were included in the analysis.

Histology scores were analysed using a Kruskal Wallis one-way analysis of variance to determine any effects of the *C. perfringens* isolate, loop treatments or time.

3.3 Results

3.3.1 *In vitro* assays

3.3.1.1 Bacterial isolate characterisation: Table 3.3. shows that both isolates used here were positive for the alpha-toxin gene; CP5 was negative for any of the other genes tested, whereas CP4 was positive for NetB, TpeL and beta-2 toxin genes.

Table 3.3. Presence and absence of *C. perfringens* toxinotyping and virulence genes in CP4 and CP5

Toxin Gene	Virulent CP4	Avirulent CP5
NetB	+	-
Alpha	+	+
Beta	-	-
Epsilon	-	-
Iota	-	-
Beta-2	+	-
Enterotoxin	-	-
TpeL	+	-

3.3.1.2 *In vitro* cytotoxicity assay for NetB: Figure 3.2 shows the cytotoxic effect of two culture supernatant dilutions of both isolates and control LMH cells that were subjected to medium alone. LMH cells subjected to incubation with either the 1:5 or 1:25 dilution of the virulent CP4 supernatant died within the first four hours following the addition of the supernatant. The

addition of the avirulent CP5 supernatant in the cell cultures at the same dilutions did not affect cell viability until around 24 hours following the addition. Control cells continue to proliferate in the sixty hour period monitored. Cytotoxicity at a dilution of at least 1/16 is consistent with the presence of functional NetB (Keyburn et al., 2008) and our data confirm this.

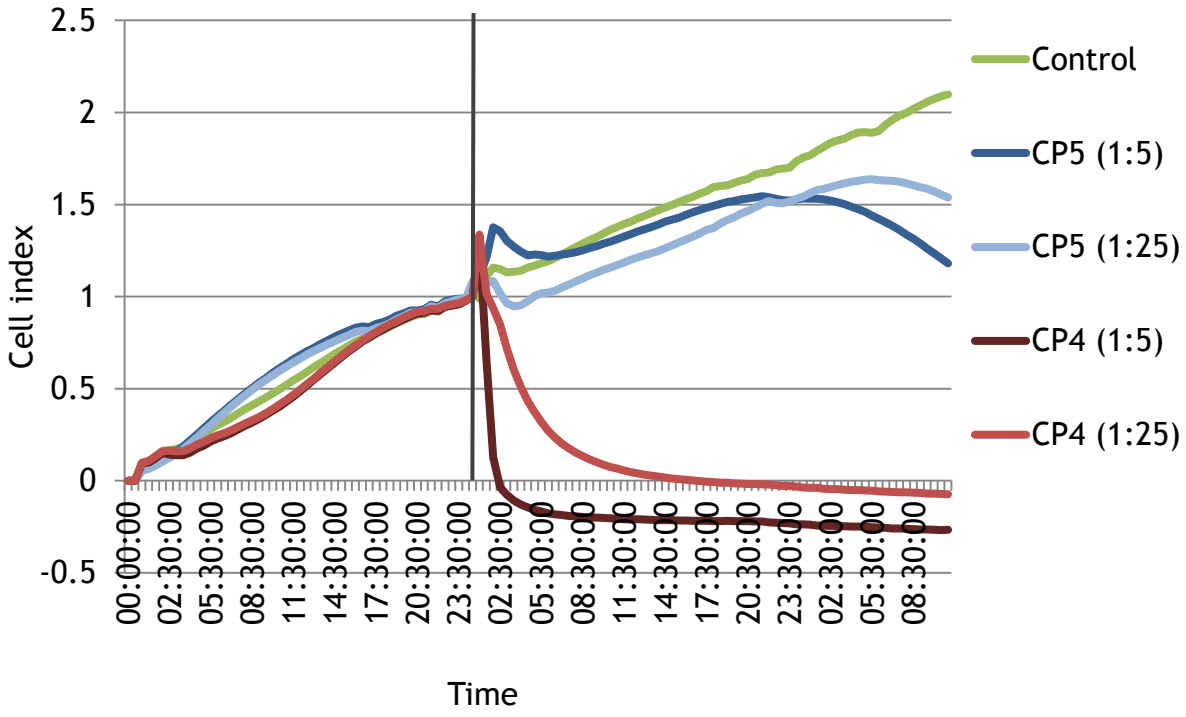

Figure 3.2. Cytotoxicity assay for NetB using RTCA

Comparison of CP4 (red lines) and CP5 (blue lines) culture supernatants on LMH cell viability. The vertical black indicates the addition of toxin at 24hours after normal cell growth. Numbers within brackets indicate the dilution of culture supernatant.

3.3.2 *In situ* experiment

3.3.2.1 Histological examination: *C. perfringens* isolate had no effect on the pathology scores in this study (p=0.562). At 0.5h post infusion, very few sections deviated from score 1 indicating that initial surgery did not alter the structure and integrity of the intestine immediately post-surgery. Sections taken from the 4h loops showed mostly score 3 across treatments indicating a significant increase in intestinal damage as time progressed (p<0.001). There was no difference detected as a consequence of the loop treatments in comparison to the control loops (p=0.395 and 0.747) (Figure 3.3).

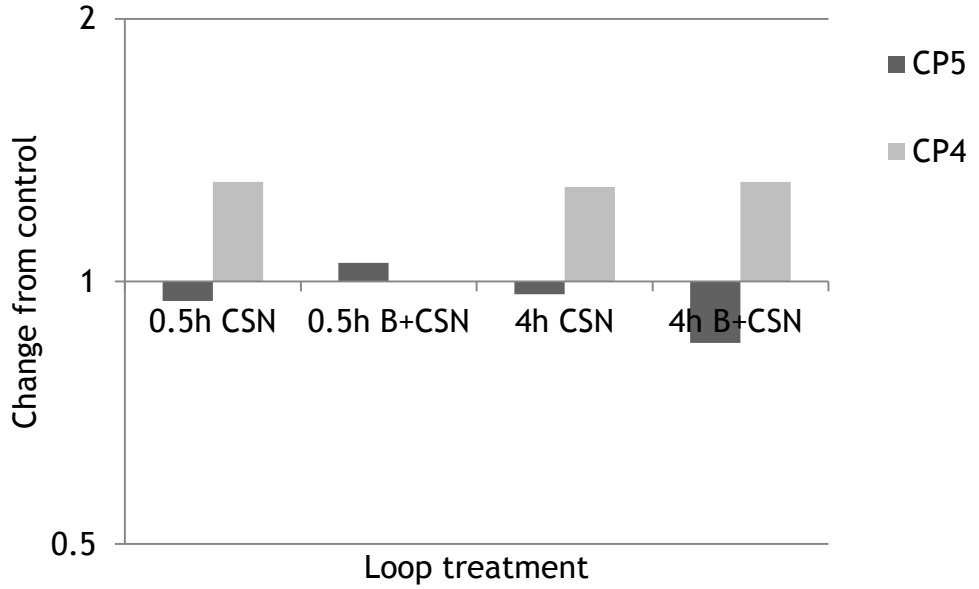

Figure 3.3. Histology Scores

Histology scores are presented in comparison to the control loops from the same bird.

3.3.2.2 Heterophil quantification: Heterophil numbers were not affected by the isolate inoculated (p=0.29) but were significantly higher in loops containing bacteria + culture supernatant (p=0.011) in comparison to culture supernatant alone and the control (Figure 3.4). Power analysis indicated that a further 73 samples would be required to determine differences between the isolates. There was a tendency for heterophil numbers to be increased over time, with more cells being present at 4h than 0.5h (p=0.089). To confirm this tendency a further 71 samples would be required. There were no significant interactions between treatments.

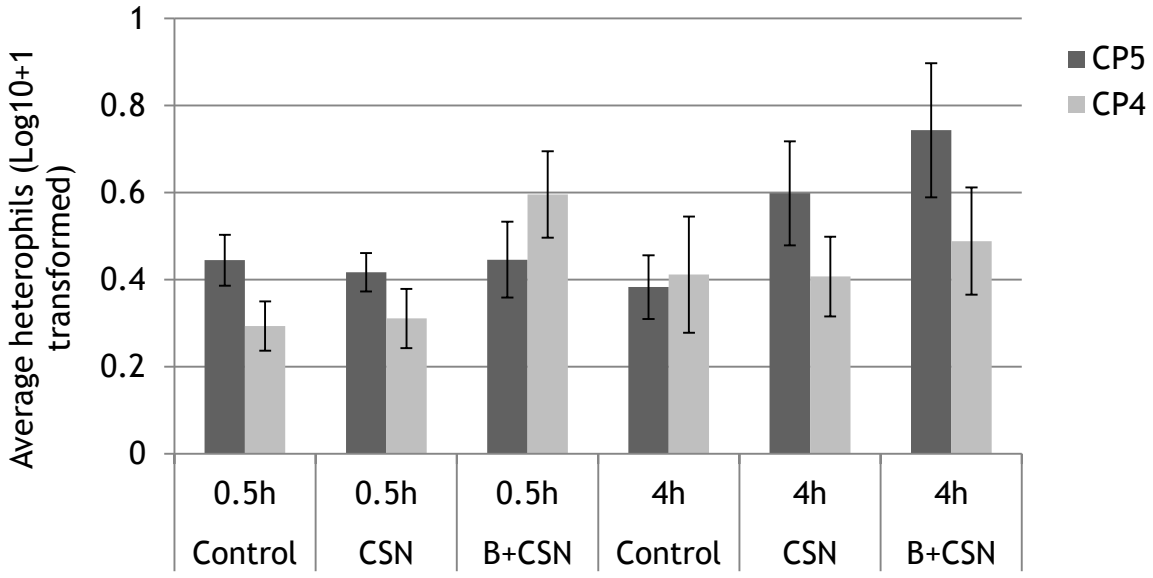

Figure 3.4. Average heterophil numbers

Log10+1 transformed data presented. Heterophils were counted in hematoxylin and eosin stained sections. Graph shows average log10+1 transformed data ±SE.CSN= culture supernatant. B+CSN=bacteria+ culture supernatant.

3.3.2.3 Normalisation gene analysis: The geometric mean of the two genes chosen by Genorm was analysed by ANOVA for differences across the experiment. There was a significant interaction detected in the control loops with the geometric mean being increased at 4h compared with 0.5 h ($p=0.006$). There was no significant effect of the isolates used ($p=0.73$) or the loop treatments ($p=0.25$).

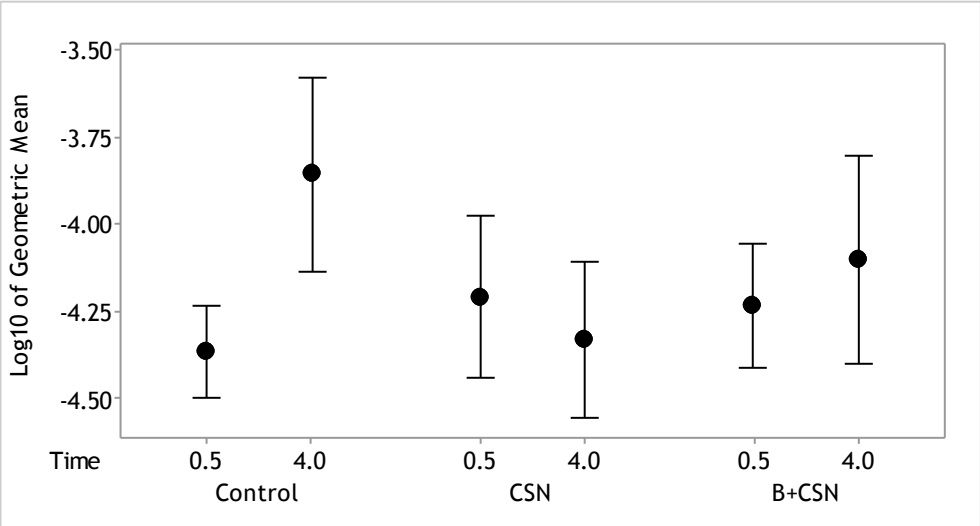

Figure 3.5. Reference gene expression (Loop x Time interaction)
The geometric mean data was log10 transformed and analysed with ANOVA which indicated a significant increase in the reference genes used at 4h compared with 0.5h in the control loops.

3.3.2.4 Gene Expression analysis: The only significant three-way interaction was detected in *IRAK-4* mRNA expression. Increased levels of this gene were detected at 0.5h in loops containing CP4 bacteria + culture supernatant. Irrespective of the bacterial isolate, there were significant treatment x time interactions, where mRNA expression of *IL-6*, *IFN-γ* and *IL-1β* were increased at 0.5h post infusion in loops containing bacteria +culture supernatant ($p=0.0002$, $p<0.0001$, $p=0.0002$ respectively). On the other hand, mRNA expression of *FAS* was increased at 4h post bacteria + culture supernatant infusion ($p<0.0001$). The presence of bacterial cells reduced *BCL6* and *B-LA* mRNA expression at 4h compared with control and culture supernatant alone.

The only host response, measured here, significantly affected by the bacterial isolate was *IL-6* mRNA expression which was reduced in the presence of CP4 ($p=0.048$) compared to CP5 across treatments. The presence of bacterial cells increased *NBL1* mRNA expression ($p<0.0001$), whereas *GIMAP8* expression was reduced in loops containing culture supernatant and bacteria + culture supernatant in comparison to the controls ($p<0.001$) (Table 3.4.)

There was no effect of the isolate on mucin gene expression. However, the presence of bacteria induced an increased expression of *MUC2* and *MUC5ac* mRNA at 4h ($p=0.001$). The absence of bacterial cells in culture supernatant loops induced *MUC13* mRNA expression ($p=0.002$) (Figure 3.5.).

Table 3.4. Mean log transformed values of gene expression data after infusion of *C. perfringens* Culture supernatant (CSN) and bacteria+culture supernatant (B+CSN)

Isolate	Loop Treatment	Time	IL-6	IFNγ	IL-1β	BCL6	IRAK4	GIMAP8	FAS	BLA	NBL1
CP4	Control	0.5h	0.0041	0.00121	0.0033	0.577	0.000131	0.739	-1.815	0.193	3.423
		4h	0.0119	0.00061	0.0069	2.165	0.000158	0.587	-0.087	1.222	2.64
	CSN	0.5h	0.0209	0.0005	0.0042	0.516	0.000268	0.281	-1.798	-0.062	3.626
		4h	0.0064	0.00038	0.0353	2.639	0.000611	0.626	-0.791	1.381	3.106
	B+CSN	0.5h	0.1334	0.00259	0.1009	0.017	0.001839	0.305	-1.708	-0.449	3.56
		4h	0.01	0.00013	0.0237	2.00	0.000165	0.208	0.963	-1.02	3.016
CP5	Control	0.5h	0.0034	0.00134	0.0091	0.569	0.000197	0.583	-1.856	0.088	3.227
		4h	0.0255	0.00086	0.0225	2.027	0.000143	0.586	-0.021	1.132	2.699
	CSN	0.5h	0.0255	0.00052	0.003	0.479	0.000279	0.387	-1.72	0.346	3.457
		4h	0.0415	0.00046	0.1113	2.576	0.000395	0.398	-0.668	1.45	3.18
	B+CSN	0.5h	0.2287	0.00294	0.0647	0.011	0.000983	0.357	-1.661	-0.343	3.66
		4h	0.0739	0.00009	0.0275	2.012	0.000244	0.252	0.942	-1.047	3.14
	P value*	Isolate	0.048	0.692	0.42	0.502	0.014	0.835	0.463	0.168	0.988
		Loop treatment	<.001	<.001	0.013	<.001	<.001	<.001	<.001	<.001	<.001
		Time	0.007	<.001	0.569	<.001	<.001	0.989	<.001	<.001	<.001

Table 3.5. Main effects and interaction output of statistical models for qPCR data

Gene	Main Effects						Interactions							
	Isolate		Loop treatment		Time		Isolate x Loop treatment		Isolate x Timepoint		Loop treatment x Time		Isolate x Loop treatment x Time	
	SED	P	SED	P	SED	P	SED	P	SED	P	SED	P	SED	P
IL-6	0.017	0.048	0.021	<.001	0.015	0.007	0.030	0.206	0.022	0.88	0.028	<.001	0.039	0.674
IFN-γ	0.000	0.692	0.000	<.001	0.000	<.001	0.000	0.937	0.000	0.881	0.000	<.001	0.001	0.888
IL-1β	0.013	0.42	0.014	0.013	0.012	0.569	0.021	0.181	0.018	0.088	0.021	<.001	0.030	0.532
BCL6	0.058	0.502	0.066	<.001	0.054	<.001	0.096	0.839	0.079	0.671	0.093	<.001	0.134	0.846
IRAK-4	0.000	0.014	0.000	<.001	0.000	<.001	0.000	0.099	0.000	0.201	0.000	<.001	0.000	0.009
GIMAP8	0.144	0.835	0.069	<.001	0.055	0.989	0.164	0.611	0.154	0.57	0.096	0.079	0.190	0.185
FAS	0.056	0.463	0.042	<.001	0.047	<.001	0.075	0.498	0.073	0.759	0.071	<.001	0.110	0.742
BLA	0.042	0.168	0.090	<.001	0.104	<.001	0.112	0.187	0.113	0.47	0.156	<.001	0.213	0.787
NBL1	0.092	0.988	0.086	<.001	0.071	<.001	0.135	0.524	0.116	0.227	0.122	0.344	0.183	0.756

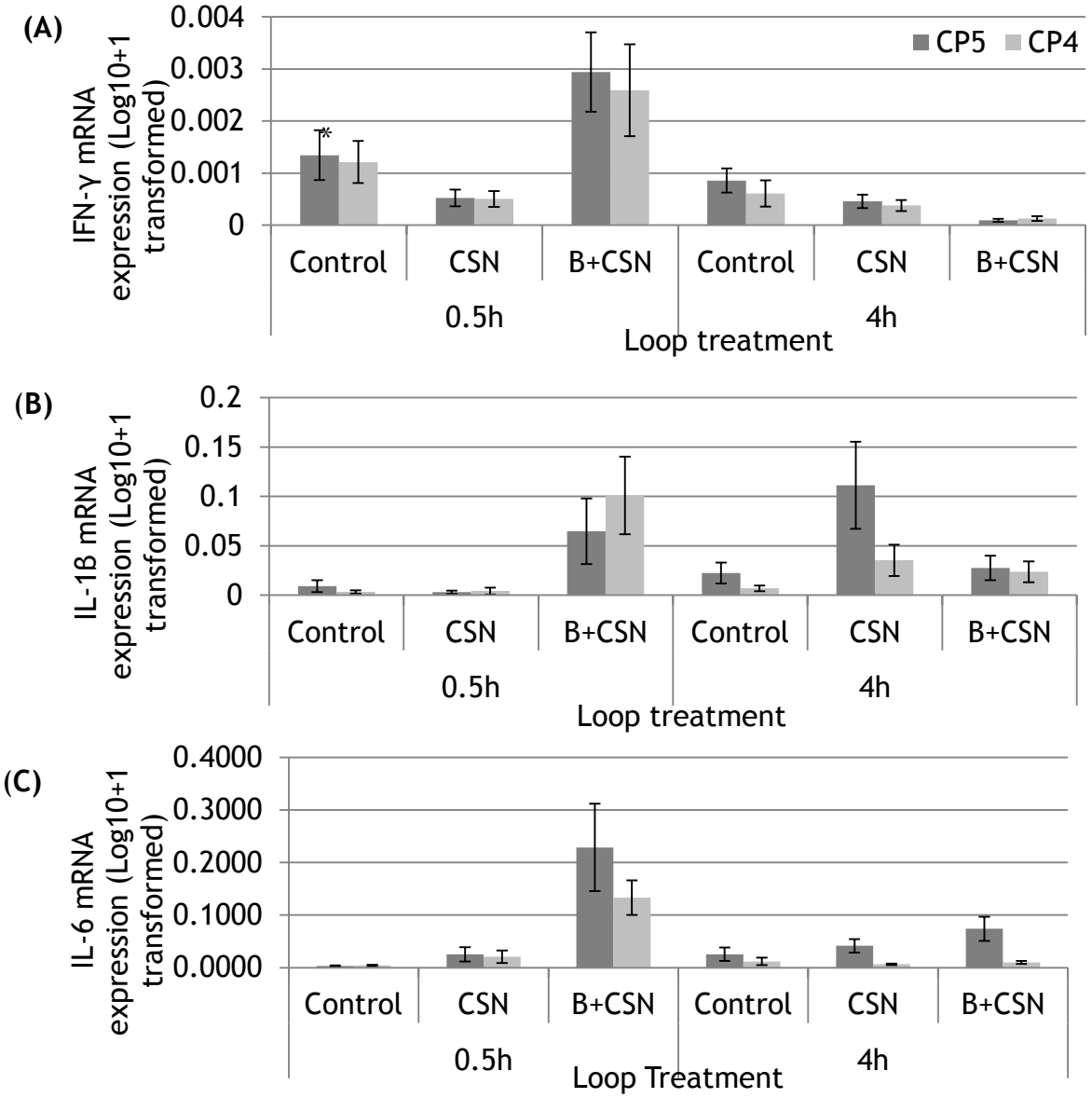

Figure 3.6. mRNA expression of pro-inflammatory genes

IFNγ (A), IL-1β (B) and IL-6 (C) expression post infusion of culture supernatant and bacteria + culture supernatant in broiler duodenal loops.

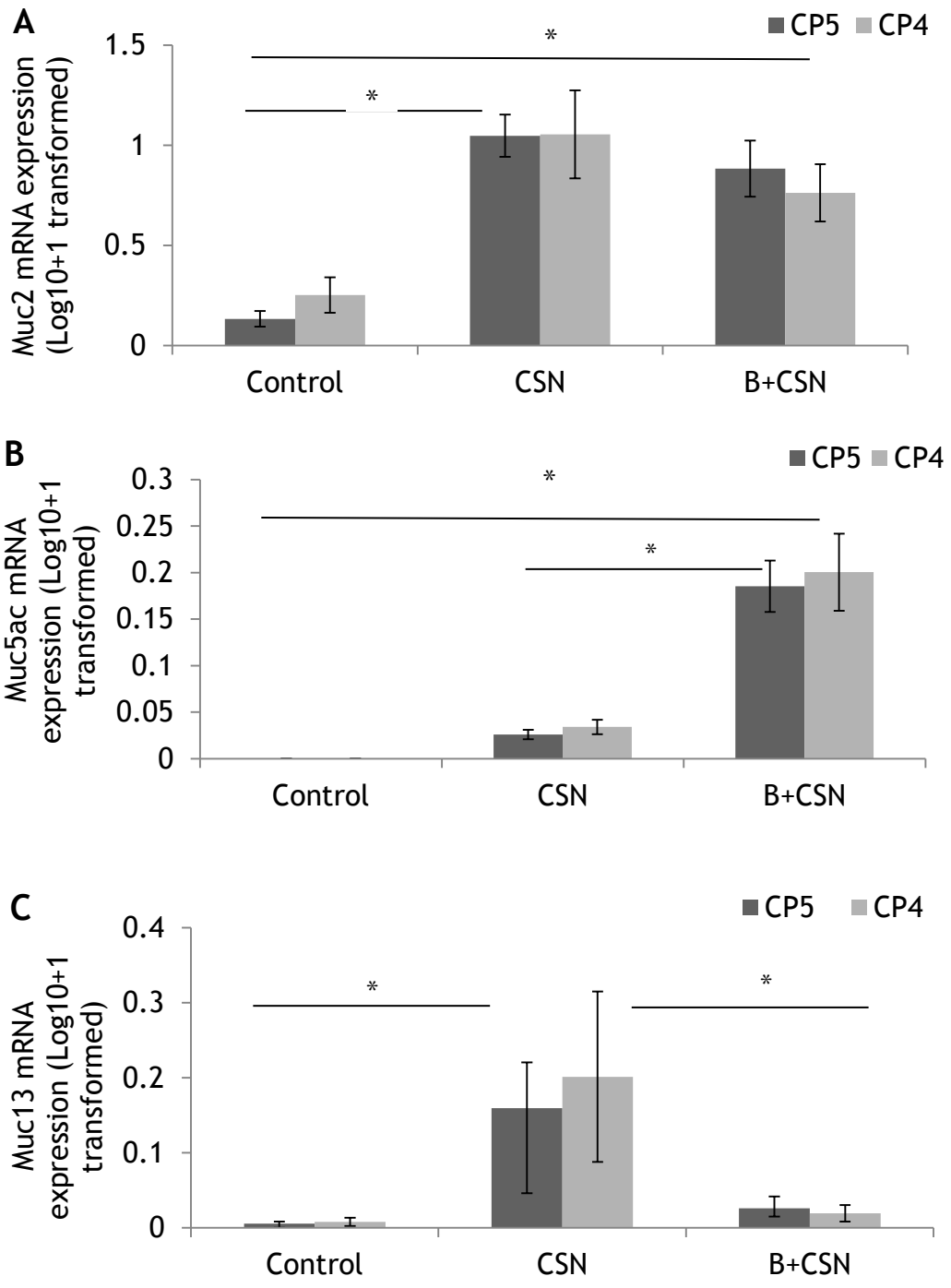

Figure 3.7. Mucin mRNA expression four hours post infusion.

Muc2 (A), Muc5ac (B) and Muc13 (C) mRNA expression at 4 hours post infusion. CSN= culture supernatant. B + CSN = bacteria + culture supernatant. *indicates significant differences between loop treatments.

3.4 Discussion

By using the *in situ* model we have been able to determine early differential gene expression patterns following exposure to isolates of *C. perfringens* that vary in their virulence over time. We were also able to identify altered patterns of mRNA expression in the presence or absence of bacterial cells over time. Although the isolates used here have different disease inducing capabilities, there was little evidence suggesting that the immune response instigated may combat them in a different manner.

Our results also indicate that virulent *C. perfringens* isolates may somehow subvert the immune system; furthermore the presence of bacterial cells seems to be required to instigate stronger host responses compared with culture supernatant alone. Although some experimental challenge models have previously compared host responses to *C. perfringens* isolates that vary in their disease inducing capabilities, to our knowledge this is the first time such responses are monitored within hours of exposure.

We have confirmed that the isolates used here differed in some of the virulence genes known to induce more severe NE in broilers with standard PCR (Brady et al., 2010; Coursodon et al., 2012). Culture supernatant from each of the two *C. perfringens* isolates used in this experiment varied in their cytotoxicity, as demonstrated *in vitro*. Our results showed that culture supernatant from CP4 induced cell death in the first four hours whereas a reduction in growth of CP5 did not occur until after 24h. Culture supernatant that is cytotoxic to LMH cells at a dilution greater than 1:8 is considered to be positive for the NetB toxin (Smyth and Martin, 2010). Based on evidence

from the LMH cytotoxicity assay, we conclude that the CP4 culture supernatant used in this work was NetB positive whereas the CP5 culture supernatant was not. Despite the differences observed in their cytotoxic properties, there were no significant differences in the pathology scores or the heterophil counts attributed to the bacterial isolate.

The presence of a NetB negative, TpeL positive *C. perfringens* type A isolate increased heterophil numbers in the duodenum and jejunum of broilers following a five day experimental infection and post-mortem on the sixth day (Calefi et al., 2014). These results imply that the presence of NetB is not required for heterophil recruitment.

Even though there was no difference between isolates, increased numbers of heterophils were detected in the duodenal loops infused with bacteria + culture supernatant indicating *C. perfringens* presence may be required to induce their chemotaxis rather than culture supernatant only. Heterophils were observed when a similar intestinal model was used and samples removed one hour post culture infusion (Al-sheikhly and Truscott, 1977a). This cell type was then reduced at three hours post infusion and remained similar at five hours. Previous work from our laboratory using the *in situ* intestinal loop model showed an increase in the number of heterophils over time following infusion of culture supernatant or control preparation but here there is only a tendency for heterophils to increase over time (Athanasiadou et al., 2015). Together these results indicate that these cells likely respond to a component of the bacteria that is present on *C. perfringens* type A and not only NetB positive isolates.

The increase in the expression of genes that encode three specific cytokines, *IL-6, IFN-γ* and *IL-1β*, in the presence of bacteria 0.5h post-infusion across both isolates is indicative of initial pro-inflammatory responses. These cytokines are predominantly produced from innate immune cells early in infection to mediate inflammation. Increased levels of these cytokines have been detected following infections with other bacteria species so a rise in the mRNA for these molecules was expected (Carvajal et al., 2008; Withanage et al., 2004). This increase was not detected in loops infused with culture supernatant alone indicating that the response here is likely due to components of the bacterial cells rather than secreted components in the supernatant. *IL-6* mRNA expression in particular was lower in broilers infused with CP4 in comparison to those infused with CP5; indicating virulent isolates may impair inflammatory responses. *IL-6* mRNA could not be detected in the ileum after *C. perfringens* challenge and levels in the spleen did not respond to challenge indicating the expression of this molecule is not up-regulated by virulent isolates of *C. perfringens in vivo* (Lu et al., 2009). Our data are also in agreement with a previous *in vitro* study, where *IL-6* measured in primary chicken epithelial cells was higher in cells exposed to increasing levels of a NetB negative isolate (Guo et al., 2015) although virulent NetB positive isolate was not used in this study. The response of various cell types to *C. perfringens* and the virulence factors it produces is unclear. Further investigation directly comparing *IL-6* responses in different cell types is required before we can fully understand the role this cytokine plays in NE infections.

Increased expression in *IL-1β* in the presence of *C. perfringens* cells observed here was consistent with previous *in vitro* studies. After stimulation for short durations of time with various bacterial and viral antigens, *IL-1β* mRNA expression was increased in heterophils, dendritic cells and macrophages (Kogut et al., 2006; Wigley et al., 2006; Wu et al., 2010). This increase in mRNA expression was consistent with increased cytokine levels; higher *IL-1β* levels were measured in the serum of birds infected with NE compared to uninfected controls (Lee et al., 2014). Heterophils and other immune cells as well as epithelial cells may influence the expression of pro-inflammatory cytokines during a NE infection.

Unlike *IL-6*, *IRAK-4* mRNA expression showed an initial increase in loops containing CP4 bacteria + culture supernatant. Increased *IRAK-4* leads to the activation of transcription factors which in turn control the expression of genes encoding pro-inflammatory cytokines (Li et al., 2002; Kawai and Akira, 2007). Here, the increase in *IRAK-4* mRNA expression in the presence of virulent bacteria was only evident at 0.5h. mRNA expression of pro-inflammatory cytokines was not affected in CP4 infused loops at 4h post infusion, although this does not exclude the possibility that cytokine production, which was not measured here may have been affected.

In addition to genes related to the up-regulation of inflammation we also measured mRNA expression of genes that may inhibit inflammation. *BCL6* in mammals is a sequence specific transcriptional repressor (Chang et al., 1996) and is an inhibitor of *IL-6* expression from macrophages (Yu et al., 2005). Intestinal loops without bacteria showed increased *BCL6* expression at the 4h

time point in comparison to the 0.5h. Furthermore, increased *BCL6* mRNA expression was observed at 4h in comparison to 0.5h across all loop treatments. This increase coincided with the reduction of *IL-6* mRNA expression. It is possible that *BCL6* is playing a role in *IL-6* regulation here, reducing transcription of *IL-6* mRNA as it has been demonstrated in previous studies *in vitro*. These data are consistent with results from a previous experiment, where *BCL6* mRNA expression was found to be the highest at the same time as lower *IL-6* expression was detected (Chapter 2; Athanasiadou et al., 2015).

NBL1 is another gene identified as having inhibitory functions in the immune system. Increased levels of *NBL1* have been associated with reduced monocyte chemotaxis from the blood (Chen et al., 2004) and have been involved in chick embryonic development (Gerlach-Bank et al., 2002). Here, up-regulated levels of this gene were detected in broilers treated with the culture supernatant of both isolates as well as in loops where bacteria were present. This indicates a secreted component in the culture supernatant may be responsible for inducing the response. Culture supernatant has for some time been known to be important in the induction of NE as bacteria re-suspended in sterile media are only able to cause mild NE whereas those re-suspended in culture supernatant produced disease more typical of field cases of NE (Al-sheikhly and Truscott, 1977b). Culture supernatant and specific sub-units of *C. perfringens* culture supernatant, such as NetB toxoids, have previously been trailed as a vaccination strategy against NE infections with some success but variation between the bacterial isolates used

and dosing regimens have hindered implementation (Fernandes da Costa et al., 2013; Lanckriet et al., 2010). Investigating the interactions of *C. perfringens* and its culture supernatant with epithelial and immune cells in the intestine could provide information on which molecules are involved and how best to target long-term protection from this disease in broilers.

Bacterial interaction with toll-like receptors (TLRs) is one hypothesis for the induction of mucin synthesis during an infection. Another concept is that antigens directly stimulate goblet cells to release these molecules into the lumen (Kim and Khan, 2013). Mucin genes are differentially expressed in regions of the gastrointestinal tract normally but their production can be stimulated by pathogens (Pelaseyed et al., 2014). In the current study different factors appeared to alter expression of the three mucin genes investigated. Our data suggest that the presence of bacteria increased *MUC5AC* mRNA 4h post infusion. This is in agreement with Forder et al., (2012) who detected increased *MUC5AC* in the jejunum of broilers co-infected with *Eimeria* three days post challenge. An increase of *MUC2* mRNA was noted in all loops containing culture supernatant, irrespective of the presence of bacterial cells. This differs from other studies which previously indicated reduced or no change of *MUC2* mRNA expression when comparing infected birds to un-infected controls (Forder et al., 2012; Liu et al., 2012). *MUC13* was reduced in the presence of bacteria but increased when culture supernatant was infused. Bacterial cells may prevent the active up-regulation of this gene as it was also reduced during a co-infection model of NE (Forder et al., 2012). The role of mucins in NE is unclear but this study suggests

changes are induced early post exposure. Mucin expression was higher in birds with greater *C. perfringens* burdens in their ileum indicating that increasing these molecules may not be beneficial to the broiler (Collier et al., 2008). Further studies are required to determine which bacterial components stimulate mucin up-regulation and whether this is a beneficial response for the bird during NE infection or whether it does, in part, encourage *C. perfringens* growth.

Some of the data from this experiment indicates that there could be a mechanism by which *C. perfringens* evades the immune system. The up-regulation in expression of inhibitory genes, *NBL1* and *BCL6*, indicate a reduced monocyte and macrophage activity. This is further supported by down-regulation of mRNA expression of pro-inflammatory cytokines *IL-6*, *IL-1β*, *IFN-γ* and *B-LA*, a component of MHC class II, which is the antigen presentation molecule 4h post infusion. This combination could be indicative of reduced phagocytic cell activity by either bacterial antigens or the host response to reduce inflammation in the duodenum. Further characterisation of antigen presenting cells incubated with *C. perfringens* antigens during *in vivo* experimental infections and *in vitro* experiments would allow us to determine whether this is a bacterial evasion strategy or a host response to limit inflammation in the intestine soon after bacterial exposure.

Host responses and bacterial evasion may also influence cell death pathways. *FAS* is a member of the death receptor family and upon stimulation induces the formation of the death-inducing signalling complex (Lavrik and Krammer, 2012). *GIMAP8* is part of the GTP-ase immune-associated

proteins, a group of genes which regulate cell survival and have anti-apoptotic properties (Dion et al., 2005; Krücken et al., 2005). *FAS* expression was increased in loops infused with bacteria compared to the control and culture supernatant at 4h post infusion of the treatments. As well as this, we found the lowest levels of *GIMAP8* expression were observed in the 4h loops, in the presence of bacteria, where the highest levels of *FAS* expression were also detected. This could indicate that the presence of bacteria drives cell death via increased mRNA expression of *FAS*.

Our results showed that the presence of *C. perfringens* cells altered the expression of a number of genes in the duodenum of broilers, which are related to immune cell activity (IL-6, IFNγ, BCL6), antigen presentation (B-LA) and disease pathogenesis (FAS and GIMAP8). In the hours post exposure to bacterial antigens we detected few changes in host responses attributed to different isolates. The reduction of *IL-6* mRNA expression in virulent CP4 treated broilers and the reduction of *B-LA* in loops containing bacteria could indicate possible evasion strategies for *C. perfringens* but further work is required to determine this in the case of NE. Culture supernatant alone also modulates gene expression and immunity but some of the biggest host responses were detected in loops where bacteria were present with increased IL-6, IFNγ and IL-1β being detected at 0.5h in these loops. As subunits from culture supernatants have been trialled as vaccine candidates with limited success (Fernandes da Costa et al., 2013; Lanckriet et al., 2010; Saleh et al., 2011), it may be that in addition to culture supernatant, bacterial particles may be required to induce a stronger response. Here we used two wild type

isolates of *C. perfringens* which had different virulence profiles but it is unclear whether specific virulence factors alone, such as NetB, can contribute to the altered expression of pro-inflammatory mediators. Chapter 4 aims to determine the role NetB, in conjunction with bacterial cells may have in early responses to *C. perfringens*.

Chapter 4 : Early duodenal responses to *Clostridium perfringens* with and without NetB

4.1. Introduction

Disease pathogenesis during Necrotic enteritis in broilers is commonly associated with bacterial toxins produced by *Clostridium perfringens* type A; the etiological agent of the disease. All type A isolates produce alpha-toxin but the main virulence factor in broilers is believed to be the NetB toxin (Keyburn et al., 2008, 2006). Isolates from cases of NE are more likely to possess the gene encoding NetB whereas isolates of *C. perfringens* without it are generally found to be avirulent in broilers and unlikely to induce disease (Abildgaard et al., 2010; Brady et al., 2010; Chalmers et al., 2008). The gene encoding this pore forming toxin is present on a plasmid (Parreira et al., 2012). NetB release is controlled via a two component system which senses and then responds to the external environment (Cheung et al., 2010). NetB monomers come together on the cell membrane to form a heptamer pore (Savva et al., 2013).

Breeding for increased carcass weight over recent decades has greatly changed the broiler. Modern boilers are around five times heavier than their 1950's counterparts and the majority of this gain has come via genetic selection (Havenstein et al., 2003). Breeding for increased body weight and improved feed conversion has also led to changes in the broiler immune system over time. Lymphoid organs in modern broilers are a smaller proportion of the body weight and antibody responses are poorer than that of birds which have not been selected for growth (Cheema et al., 2003). Comparisons of different commercial broiler lines indicated those with lower body weights had increased bursa and spleen weights than heavier birds of

the same age (Rama Rao et al., 2003). Macrophages from four broiler lines have different responses *in vitro* e.g. some phagocytose bacteria better than others (Qureshi and Miller, 1991). A meta-analysis of selection experiments indicated that selecting for growth has compromised immunity in birds but in the future selecting for immune traits would not necessarily hinder growth (Van Der Most et al., 2011). This work indicates that host genetics can play a role in susceptibility or resistance to certain pathogens (Cheng et al., 2013).

There is some evidence indicating that host genetics play a role in protection to *C. perfringens* as not all broilers within disease challenge experiments will succumb to infection whereas some will score highly on lesion score scales (Cao et al., 2012; Casterlow et al., 2011; Jiang et al., 2009; Kulkarni et al., 2010). Generally, these studies use one line of commercial broiler. Breeding companies will have prioritised different traits over recent decades to gain commercially in the broiler market and it is possible that these selections could have impacted on immune traits. Here, we used the *in situ* duodenal loop model to investigate early host responses to *C. perfringens* in two commercial breeds, the Cobb and Hubbard, which could alter the induction of protective responses later in infection. We also aim to determine the role of NetB *per se* on early host responses. To achieve this we used a virulent isolate positive for NetB and a mutant isolate which was identical to the virulent one, with a mutation in the NetB gene, thus preventing the ability to produce this virulence factor.

4.2. Materials and Methods

4.2.1. *In vitro* assays

4.2.1.1. Bacterial isolate characterisation: CP1 and CP1ΔnetB::ErmRAM were used in this experiment. CP1 is a virulent isolate able to induce NE in broilers (Jiang et al., 2009), whereas ΔnetB::ErmRAM (CP1ΔnetB) was produced following the insertion of the erythromycin gene sequence into the netB gene in isolate CP1 which prevents the production of NetB during culture (Parreira et al., 2012). This isolate requires erythromycin for proliferation during culture. The genotypes of these two isolates of *C. perfringens* were confirmed with standard PCR. Beta, epsilon, iota, beta-2, enterotoxin, netB and TpeL were toxin genes were tested. The isolates were cultured from glycerol stocks on sheep blood agar plates overnight at 37°C anaerobically. One colony from each plate was transferred to 100µl of RNase/DNase free water. This was boiled for 10mins and then centrifuged at 14000g for 10 minutes for DNA extraction. The culture supernatant was removed and used for the PCR. Phire hot start II DNA polymerase (Finnzymes, Thermo Scientific) was used in 20µl reactions. Primer sequences for the isolate typing and virulence genes are shown in Table 3.1. Different NetB primers were used for these isolates to account for the genetic mutation (Forward: AACTACTTAATAGACACAGGAA, Reverse: TACAGGATCAGTATCATATACC). A shorter PCR product is amplified in wildtype CP1 (706bp) and a longer band in CP1ΔnetB (~2600) which accounts for the erythromycin gene insertion.

4.2.1.2. Culture of *C. perfringens*: Every other day, five to seven bacterial colonies from blood agar plates for both CP1 and CP1ΔnetB were transferred to 5mls of TPG broth (5% tryptone, 0.5% protease peptone, 0.4% glucose, 0.1% thioglycolic acid) and maintained at 37°C anaerobically overnight. The evening before surgery, 0.2mls of the culture, was transferred to 10mls of TPG broth (2% v/v) and kept overnight at 37°C in an anaerobic jar to gain the appropriate number of bacteria for the *in situ* experiment. All cultures of CP1ΔnetB contained 15μg/ml. Following the incubation, these cultures of each isolate were centrifuged at 14000g for 10mins. Most of the culture supernatant (CSN) was removed and was stored at -20°C to be used for *in vitro* experiments. The bacterial pellet was re-suspended in 1ml of the culture supernatant and this was subsequently used in the *in situ* experiment. Bacterial concentration used for infusion was approximately 1.5 $x10^9$ CFU/ml. Bacterial cultures for *in situ* experiments were prepared daily for each bird. 100mls of *C. perfringens* culture was prepared in the same way (2% v/v). After centrifugation the bacterial pellet was again re-suspended in 1ml of culture supernatant and used as a 10x treatment (CPx10) for the *in situ* experiment and was approximately 1.5 $x10^{10}$.

4.2.1.3. Cytotoxicity assay for NetB: To confirm the presence of functional NetB in the culture supernatant, a cytotoxicity assay based on one described by Smyth and Martin (2010) was performed (Smyth and Martin, 2010). The real time cell analyser xCelligence DP system was used to quantify adherent cell proliferation and viability as previously described in Athanasiadou et al., 2015; (and in Chapters 2 and 3). Briefly, LMH cells (Kawaguchi et al., 1987)

were maintained at 5% CO_2 and 37°C in Weymouth's MB 752/1 medium supplemented with 10% foetal calf serum, 1% chicken serum, 100 U/ml penicillin and 100 U/ml streptomycin for the duration of the experiment. xCelligence E-plates were coated with gelatin (0.1%, Embryomax solution, Millipore) to allow LMH cells to adhere. 50µl Weymouth's MB 752/1 medium was added to each well and the plate was inserted into the xCelligence to measure the background impedance. LMH cells (100, 000) were then added to each well in 50µl of medium and the E-plates were returned to the xCelligence. The cells were allowed to settle on the base of the well for 30 minutes prior to cell index readings being taken. Cell index readings were taken every 30 minutes for 24 hours. After this initial growth period the plates were removed and 100µl of a 1:5 dilution series of filter sterilised CP1 or CP1ΔnetB culture supernatant was added to the wells in triplicate starting with the undiluted supernatant. The E-plates were returned to the DP system and cell index readings were taken every 30 minutes for a further 60 hours.

4.2.2 *In situ* experiment:

4.2.2.1 *C. perfringens* challenge using an *in situ* duodenal loop model:

An *in situ* model was used as previously described (Athanasiadou et al, 2015). Briefly, male Cobb (n=10) and Hubbard (n=10) broilers were housed together to ensure same rearing conditions. Hatch dates were staggered to ensure birds were 18 days of age at the time of the procedure. Broilers were terminally anaesthetised prior to undergoing the experimental procedure, which resulted in the creation of four loops; two on either side of the duodenal loop (Athanasiadou et al, 2015). The experimental design is shown

in Figure 4.1. Each of the four loops was assigned to one of the following treatments: **i) Control**, which was infused with 0.3ml of sterile TPG broth (ten birds/breed) **ii) CP1**, which was infused with 0.3ml $1.5x10^9$ wild type CP1 (ten birds/breed) **iii) CP1ΔnetB**, infused with 0.3ml $1.5x10^9$ CP1ΔnetB::ErmRAM (ten birds/breed) and **iv) CPx10**, 0.3ml of either concentrated CP1 or CP1ΔnetB (five birds/breed). Following infusion, the duodenum was returned to the abdominal cavity; duodenal loops were removed 4h post infusion. Each loop was split into three parts then either fixed in 10% formalin for histological analysis, stored in RNA-later for gene expression analysis or snap frozen in liquid nitrogen for immunohistochemistry. The final loop, which contained CPx10 culture, was allocated across the week to ensure five birds of each breed received each isolate (Cobb CP1x10 n=5, Cobb CP1ΔnetBx10 n=5, Hubbard CP1 n=5, Hubbard CP1ΔnetB n=5). At the end of the 4h incubation period the content of these loops were used for retrieval of bacteria. The loop contents were mixed with five volumes of RNA later for bacterial transcriptomic analysis (this work will be completed by our collaborators at the University of Guelph and does not make up part of this thesis).

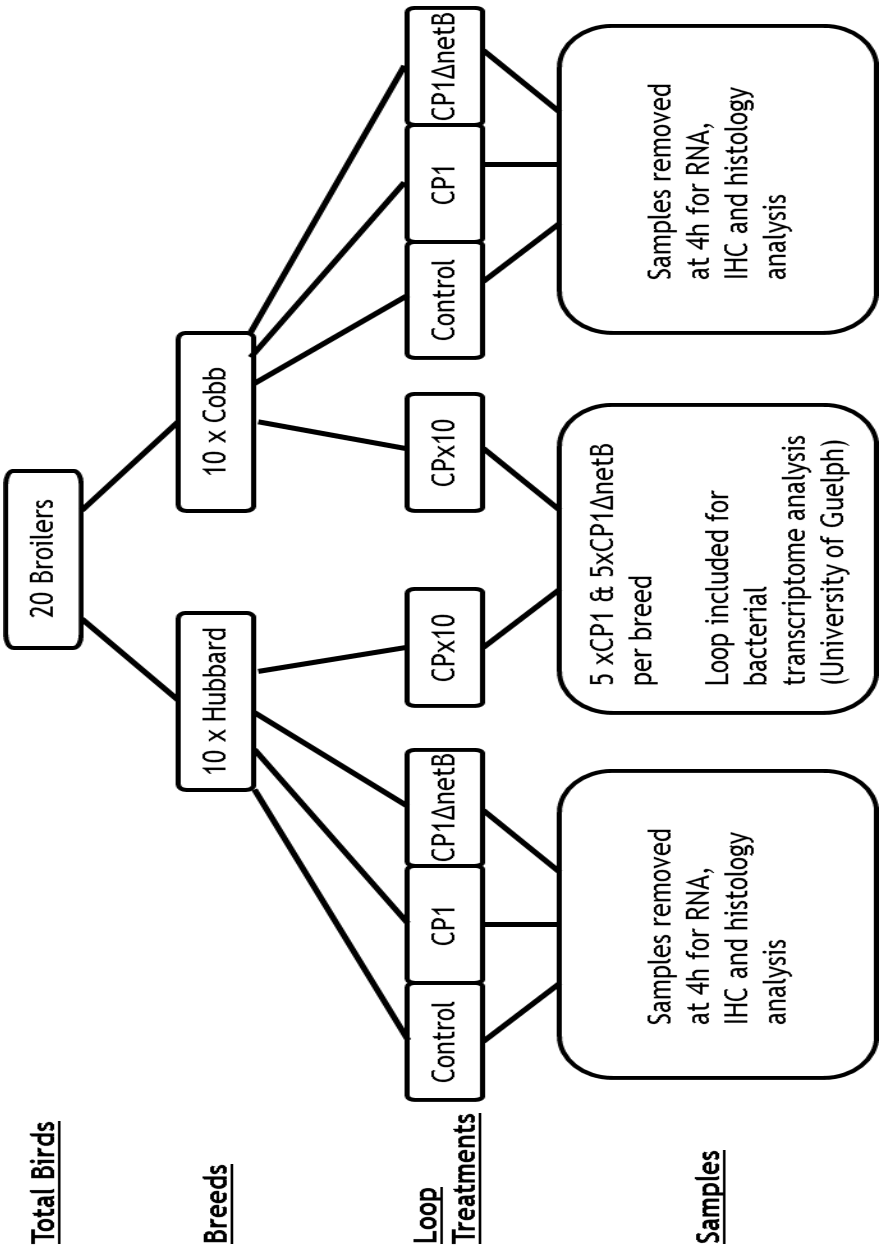

Figure 4.1. Duodenal loop experimental design

Design utilising 20 birds with 10 broilers from each breed (Cobb and Hubbard). Loops were infused with bacterial culture preparations or control and then removed four hours later for histology, immunohistochemistry (IHC) and RNA analysis. All broilers received Control, CP1 and CP1ΔnetB. (n=10/treatment/breed). The concentrated cultures were carried out five times per breed for each isolate (n=5/breed).

4.2.2.2. Histological examination: Tissue was fixed in 10% formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin (H&E). Stained sections were evaluated and scored for microscopic pathological lesions in a scale from 1 to 3 (as in Chapter 3): Score 1 indicated that there was no pathology observed. Score 2 indicated sporadic villi fusion and cell death but no crypt loss. Score 3 indicated villi fusion throughout the section and areas of complete crypt loss.

4.2.2.3. Heterophil quantification: The H&E sections used for pathology scores were also examined under x400 magnification for the quantification of heterophils. Five high powered fields were chosen throughout the section and the number of heterophils counted in each, to calculate the average number of heterophils across these fields of view. Heterophil quantification was carried out in all duodenal loops, in all birds.

4.2.2.4. Immunohistochemisty: Cryostat sections 7μm thick were cut from snap frozen tissue and placed on Superfrost® slides (Thermo), which were treated with an egg white and glycerine coating (1:1), and allowed to dry overnight. Sections were fixed in acetone for 10 minutes prior to staining protocol. Primary antibodies for macrophages (KUL01) and γδ T cells (TCR1) were diluted in phosphate buffered saline (PBS) and applied to each slide and incubated at room temperature for one hour. Slides were washed with PBS. They were then incubated with a horse radish peroxidase (HRP) conjugated goat anti-mouse antibody (Cambridge biosciences) diluted in PBS with 25% chicken serum (Life Technologies) for 30 minutes. Slides were again washed prior to colour visualisation of HRP bound antibody with Nova-red (Vector

laboratories). Following colour development, sections were washed in distilled water and counterstained with hematoxylin QS (Vector laboratories) for 5 seconds. Slides were washed in tap water and allowed to air dry overnight before mounting. Sections were examined using a Nikon (Eclipse Ni) light microscope and images were taken using Zen 2012 (blue edition). Three representative images from three different sections were captured for each sample and analysed using ImageJ software (version 1.49d). The percentage area occupied by immunostained (red) cells in each image was calculated. The average of the nine percentage areas was calculated for each sample. This was carried out for both KUL01 and TCR1.

4.2.2.5. Gene Expression analysis: Gene expression analysis was performed with qPCR. Precellys lysis tubes (Stretton Scientific, Stretton, UK) were used for homogenising previously fixed duodenal tissue with the RNeasy kit (Qiagen) to extract RNA from the tissue (used to manufacturer's specifications). RNA was converted to cDNA using a Verso cDNA kit (Thermo Scientific) and was stored at -20^{0}C. Quantitative PCR was carried out using an Mx3000 thermocycler (Stratagene). Brilliant III Ultra-Fast Sybr Green qPCR Mix or Brilliant III Ultra-Fast qPRC Mix (Agilent) was used with 1µl of cDNA (diluted 1:10 in nuclease free H_2O) in a 20µl reaction. The thermal cycle was 95^{0}C for 3 minutes and then 40 cycles of 95^{0}C for 20 seconds then 20 seconds of the primer specific annealing temperature as shown in Table 4.1. Purity of the PCR product was determined using a melting curve. Standard PCR conditions were used to obtain the product of each amplicon. PCR products were purified and quantified using a Nanodrop™

spectrophotometer (Thermo Scientific) and used to produce standard curves for the determination of relative concentrations. PCR products were sequenced to verify that correct products were amplified. PCR products were diluted to produce top standards which were detectable during qPCR amplification at around 14-16 cycles, with seven ten-fold serial dilutions forming the standard curve (Gong et al., 2010). Expression values of target genes were normalised to the reference genes, YWHAZ and 28S. Their geometric mean was calculated and used for normalisation. These genes were selected from a panel of genes in the Gallus gallus 6 gene geNorm kit (PrimerDesign Ltd) and selected as the least variable by genorm analysis in Qbase plus software. Target genes related to immune cell activity were selected based on results from previous experiments which investigated host responses to *C. perfringens* culture supernatant alone (Athanasiadou et al., 2015) or isolates with different virulence profiles (Thesis Chapter 3). Additional target genes involved in pro-inflammatory responses mediated by KUL01 and TCR1 positive cells were also investigated.

Table 4.1. Primer sequences for qPCR

RNA target	Sequence		Annealing temp (°C)
FAS	For	CCTGACCCACCACGTCCCTGA	60
	Rev	GGTTTCGTAGGCTCCTC	
IRAK4	For	TGGCAGAAACGTGGCTGTCAAGA	65
	Rev	ACCAAACAGGGCTGAGCACCATC	
B-LA	For	ACGTCCTCATCTGCTACGCCGA	60
	Rev	TTCCGGCTCCCACATCCTCTGG	
NBL1	For	CGGCTGCGAGTCCAAGTCCATC	60
	Rev	TCCACCAGCTTGTCAACCCTGG	
GIMAP8	For	TCGTGGGCAAGACGGGGAGT	65
	Rev	CCGCAGAAGCGGCCTTTAGC	
BCL6	For	CCCCAAGCGAGCAGACTCAACAAC	60
	Rev	AGGCTGAGCCAGAGGTGTGAA	
IL-6	For	TGTGCAAGAAGTTCACCGTGT	60
	Rev	TTCGTCAGGCATTTCTCCTCGT	
IL-1β	For	GTGAGGCTCAACATTGCGCTGTA	65
	Rev	TGTCCAGGCGGTAGAAGATGAAG	
IFN-γ	For	ACACTGACAAGTCAAAGCCGC	65
	Rev	AGTCGTTCATCGGGAGCTTG	
IL-10	For	GGCTGTGAAATGCAGATGTAAG	60
	Rev	GCTTCCTGACGGGTGATATAAA	
Muc2	For	GCAGCCTTATCCTGAGTGAAATC	60
	Rev	CAGGCATCATGAACACAAGCA	
	Probe	TTTGTCATTCCAAGGTGAACCCATCTCC	
Muc5AC	For	CTGAATGTCACTCAACAGTGAA	60
	Rev	CAGTGTTCTCACAGTTACATGT	
	Probe	CAGAAGTTTACCAGAAGAACTGCATGTTTG	
Muc13	For	GCAGTAAGGACAGCAGCAGAA	60
	Rev	TTGCATCATCACATGGATAAACA	
	Probe	TGCGTCAGTACGCAAATACATCCCAATGT	
28s	For	GGCGAAGCCAGAGGAAACT	60
	Rev	GACGACCGATTTGCACGTC	
	Probe	AGGACCGCTACGGACCTCCACCA	

Primers for YWHAZ were purchased from Primer Design Ltd and are proprietary and not disclosed as part of the Gallus gallus genorm kit.

4.2.3. Statistical analysis: Bird body weights were analysed using ANOVA in Minitab (Version 17) to compare differences of the two commercial breeds at day 18 of age. Linear mixed-effect models were performed using lme from the 'nlme' package (v 3.1-120) in the statistical programme R (v 3.1.2 (c) 2014 The R Foundation for Statistical Computing). The experiment was set up as a 2x3 factorial where the breed (Cobb and Hubbard) and loop treatments (control, CP1 and CP1ΔnetB) were entered into the model as fixed effects, and the bird I.D. was included as a random effect, to take into account that responses from the same bird were related. Initially a maximal model was run with all pair-wise and 2-way interactions included. Step-wise deletion of non-statistically significant terms was then performed sequentially, with the end result being a minimal model for the data of only statistically significant interactions and associated main effects. $P<0.05$ was set to indicate statistical significance throughout. Data from the heterophil quantification and mRNA expression analysis were transformed (Log10) to normalise the variance prior the statistical analysis. Pathology scores were analysed using a Kruskall Wallis one-way ANOVA to determine the treatment effects. Data from the RTCA cytotoxicity was analysed using a repeated measures ANOVA to determine the effects of both culture supernatants in comparison to the control over time.

4.3. Results

4.3.1. *In vitro*

4.3.1.1. Bacterial isolate characterisation: PCR was carried out on both isolates from the University of Guelph to confirm genetic characteristics. Both isolates were only positive for the alpha-toxin gene from the toxinotyping genes confirming both were Type A. Both were positive for NetB but the larger band was present in CP1ΔnetB indicating the additional insert to this gene.

Table 4.2. PCR characterisation of CP1 and CP1ΔnetB

Gene	CP1	CP1M
Alpha	+	+
NetB	+ (706 bp)	+ (~2600)
TpeL	-	-
ß2	+	+
Enterotoxin	-	-

4.3.1.2. Cytotoxicity assay for NetB: Culture supernatants from each isolate were added to the LMH cell to determine the presence or absence of the NetB toxin. Undiluted wild type CP1 culture supernatant reduced the cell index soon after the addition to LMH cells and after 2h all cells were dead. As CP1 was serially diluted 1:5 the effect on LMH cells lessened and at a dilution of 1:625 there was no difference from cells incubated with medium 12h after the addition of supernatant. Undiluted CP1ΔnetB also reduced the cell index

of LMH cells but not to the extent of CP1. Some cells incubated with undiluted CP1ΔnetB culture supernatant were still present after the duration of the experiment (dark green on graph). Again, serial diluting CP1ΔnetB culture supernatant reduced the effect and may even stimulate growth of this chicken cell line when diluted 1:25 as a significant increase in cell index was detected. (Figure 4.2).

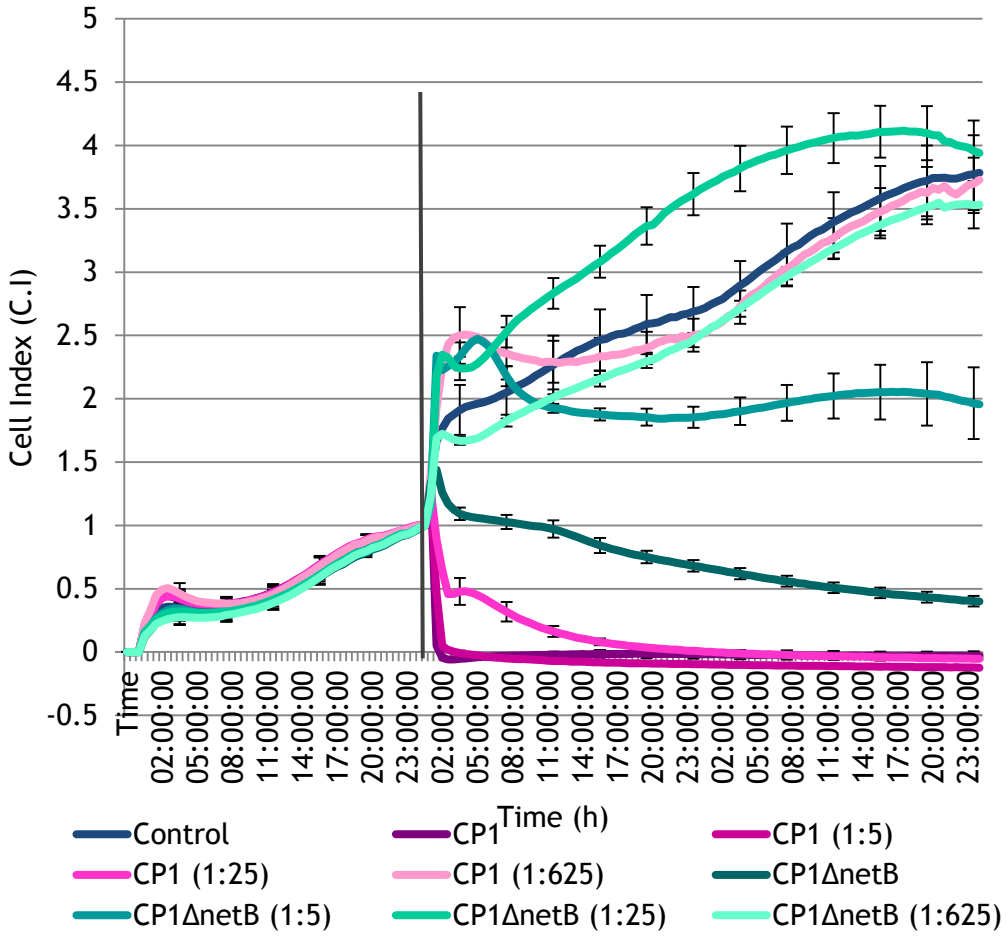

Figure 4.2. Cytotoxicity assay for NetB

Comparison of CP1 and CP1ΔnetB culture supernatants on LMH cell viability. The vertical black indicates the addition of toxin after 24h of cell growth. Numbers within brackets indicate the dilution factor. SE presented at 4hourly intervals.

4.3.2. *In situ*

4.3.2.1. Broiler body weights at time of surgery: All broilers were 18 days old at the time of surgery with weights ranging from 220g to 580g. Cobb broilers had a significantly larger body weight at D18 in comparison to the Hubbard birds of the same age ($p<0.001$). Figure 4.3 shows the range of weights from each breed along with the mean.

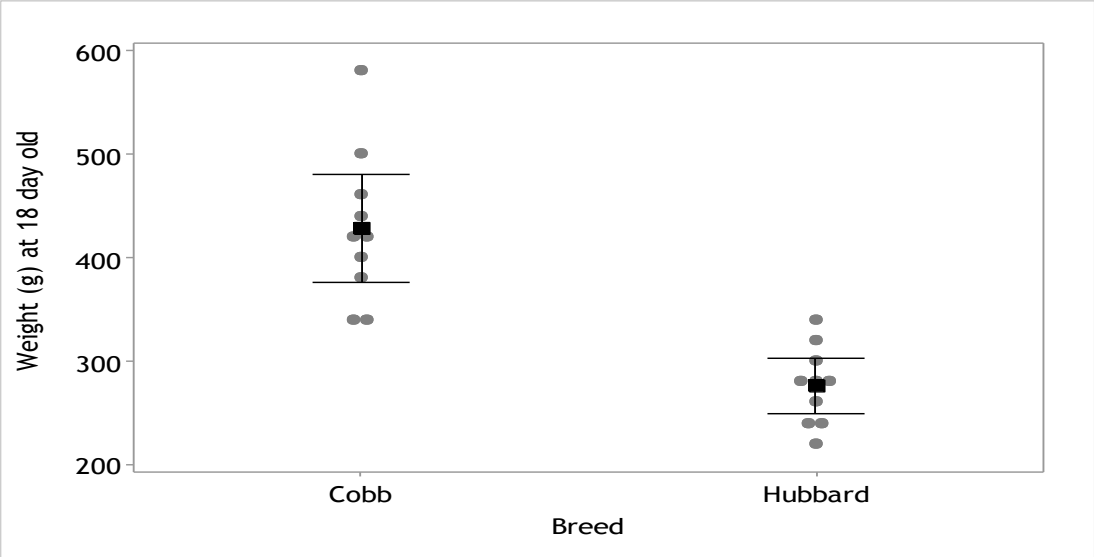

Figure 4.3. Broiler body weights

Cobb broilers were significantly heavier than their Hubbard counterparts at the same age. Cobb broilers were 428g on average whereas Hubbard broilers were 276g.

4.3.2.2. Histological examination: Pathology scores were similar in segments of duodenal chambers of Cobb and Hubbard broilers ($p=0.615$). Cobb birds averaged a pathology score of 1.9 across the three loop treatments and Hubbard scored 2.0 on average. Similarly, there was no significant treatment effect between control loops and loops containing either CP1 ($p=0.570$) or CP1ΔnetB ($p=1$) bacteria.

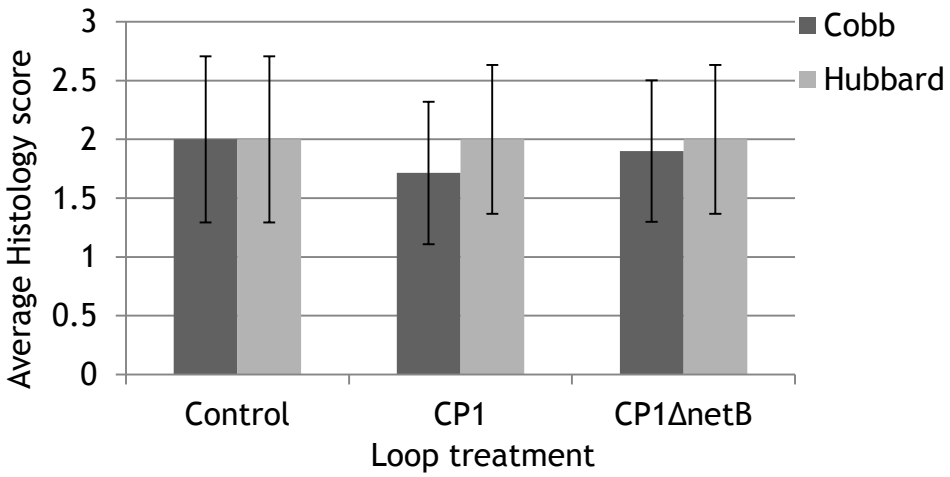

Figure 4.4. Histology score

Histology scores were evaluated from H&E stained sections. Average histology scores for each loop treatment in both commercial breeds ± SE. n=10 for each group.

4.3.2.3. Heterophil quantification: Heterophils were present in all sections evaluated. There was no significant difference observed in the number of heterophils present in broilers of the two breeds (p=0.91). Similarly, the number of heterophils was not affected by the loop treatment infused in the duodenum (p=0.45). The average heterophil count in both breeds for each loop treatment is indicated in Figure 4.4.

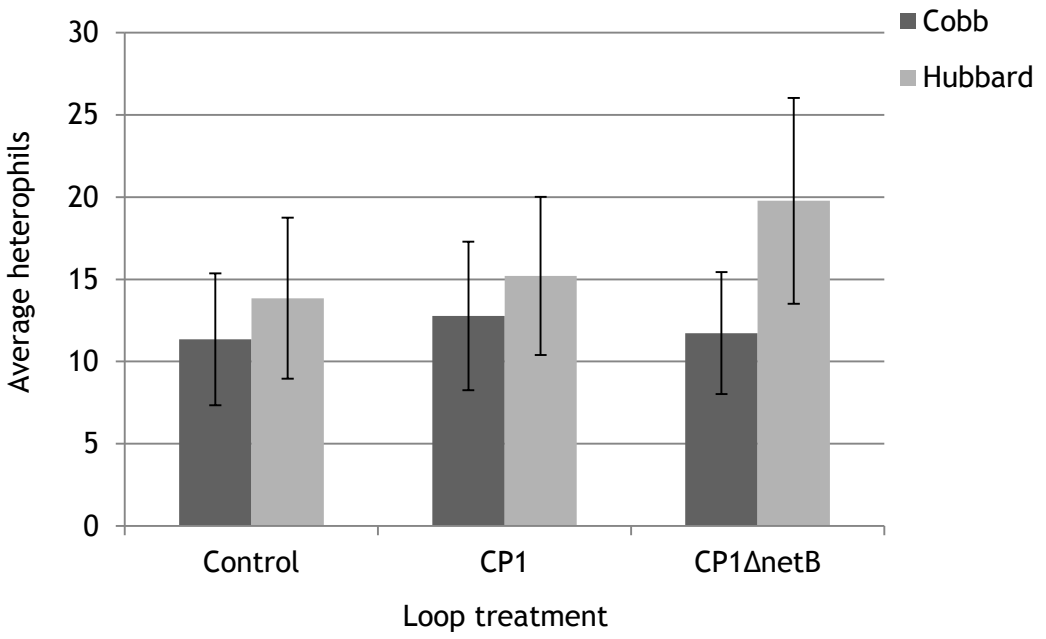

Figure 4.5. Mean heterophil count

Heterophils were counted in five high powered fields in H&E stained sections. The mean ± SE is presented. n=10 for each treatment.

4.3.2.4. Immunohistochemistry: A significantly larger positive area was identified in Hubbard broilers with the KUL01 marker in comparison to Cobb broilers (p=0.0063). There was a significant breed x treatment interaction with Cobb broilers showing a smaller positive area stained with KUL01 following infusion with the avirulent CP1ΔnetB isolate compared to loops infused with virulent CP1 (p=0.02) (Figure 4.5). Similarly, there was an increase in the TCR1 marker in duodenal sections from Hubbard broilers compared to Cobb birds (p=0.001). Loop treatment did not affect the positive area of γδ T cell staining (p=0.864) (Figure 4.6).

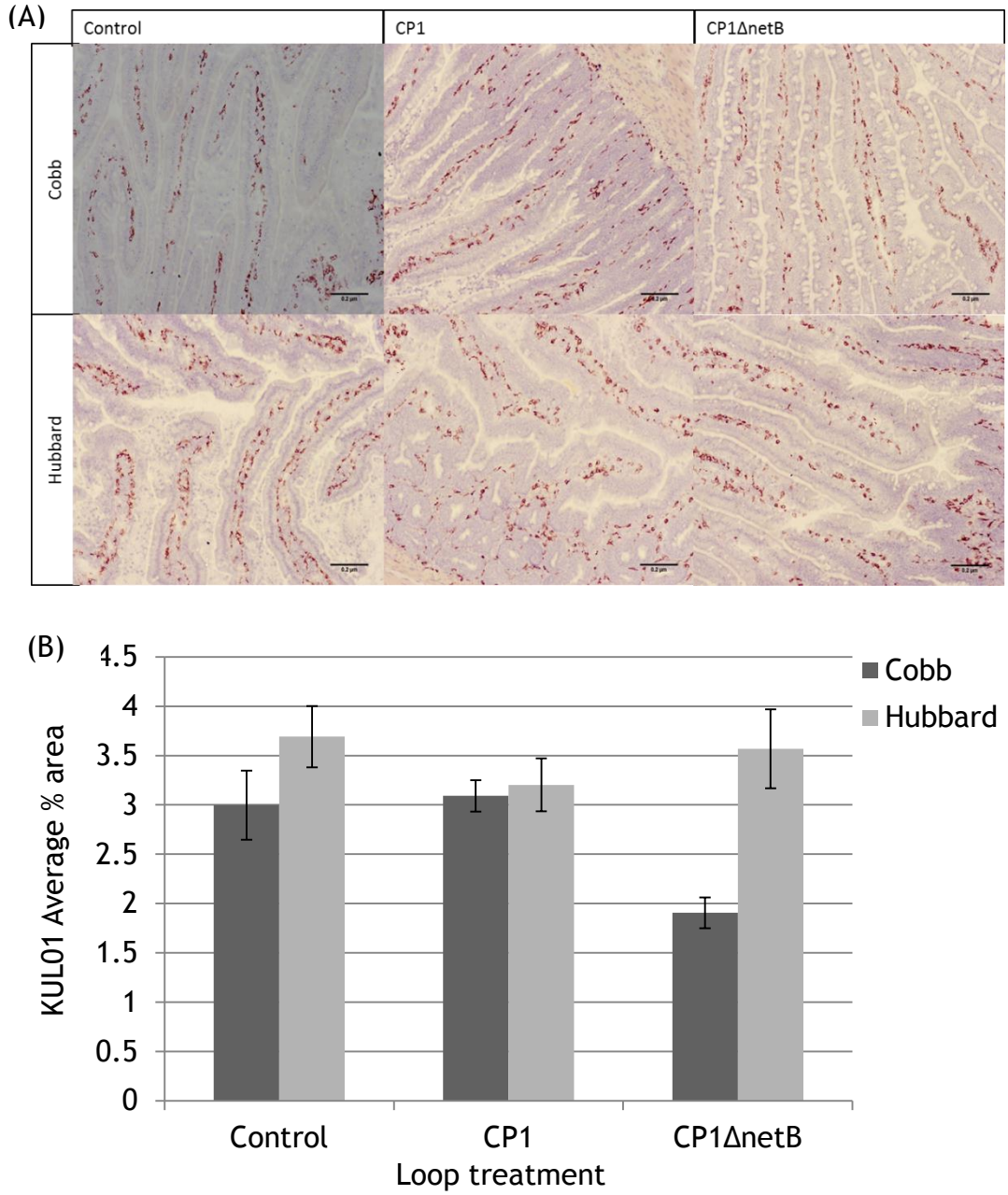

Figure 4.6. KUL01 in duodenal sections.

KUL01+ cells stained red in sections from duodenal loop model. Images were taken at x100 magnification (A). Graph indicates the average percentage area determined from nine high-powered (x400) images (three images from three different sections per loop treatment) (B).

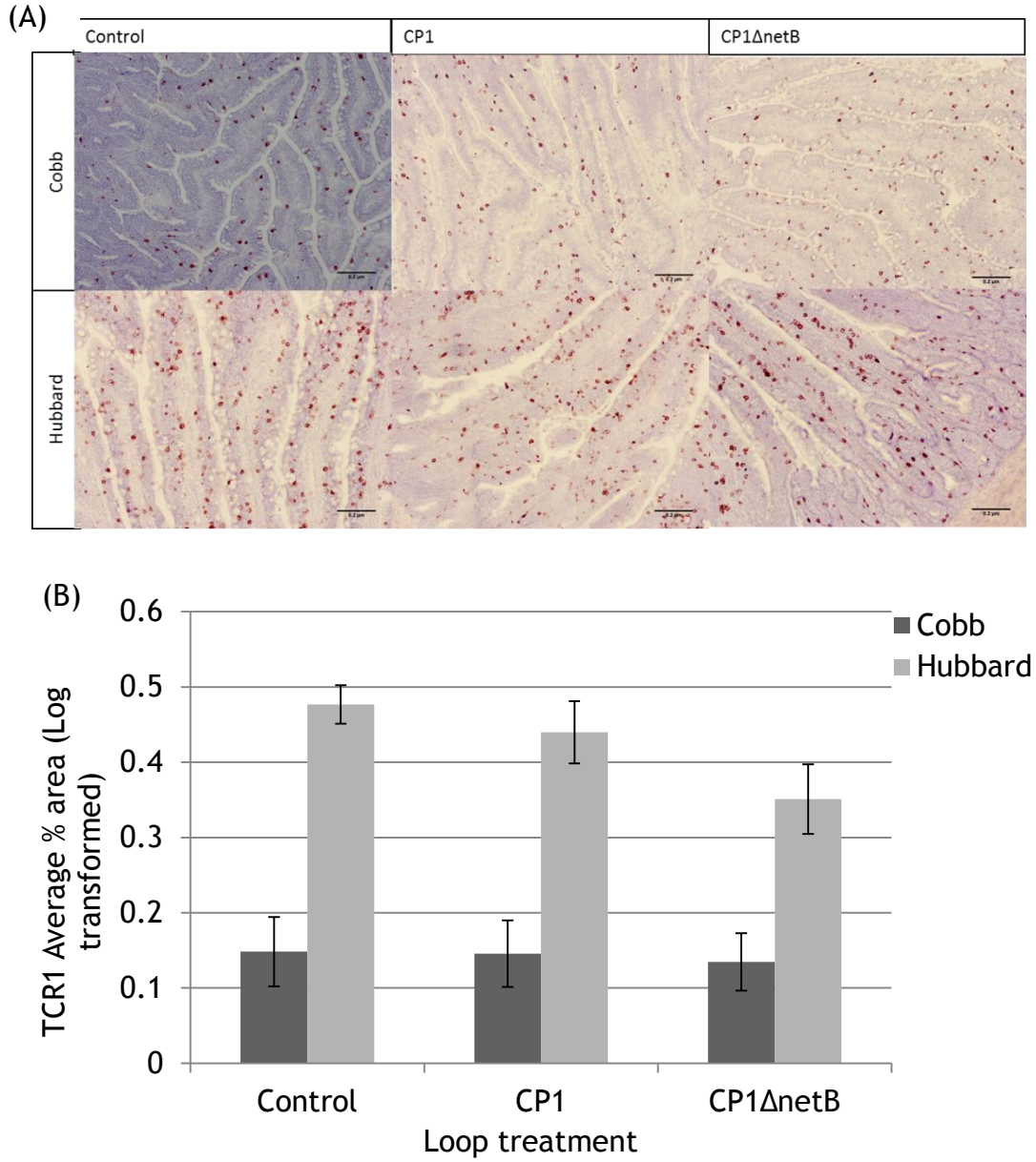

Figure 4.7. TCR1 in duodenal sections

TCR1+ cells stained red in sections from duodenal loop model. Images were taken at x100 magnification (A). Graph indicates the average percentage area determined from nine high-powered (x400) images (three images from three different sections per loop treatment) (B).

4.3.2.5. Analysis of normalisation genes

The geometric mean of the reference genes was analysed by ANOVA for differences across the experiment. There was no significant interaction detected across the three loop treatments analysed (p=0.96). There was a significant effect of the isolate used with CP1 loops having an increased level of the normalisation genes overall (p=0.003) compared with the control or CP1M. There was no difference across the two breeds (p=0.93).

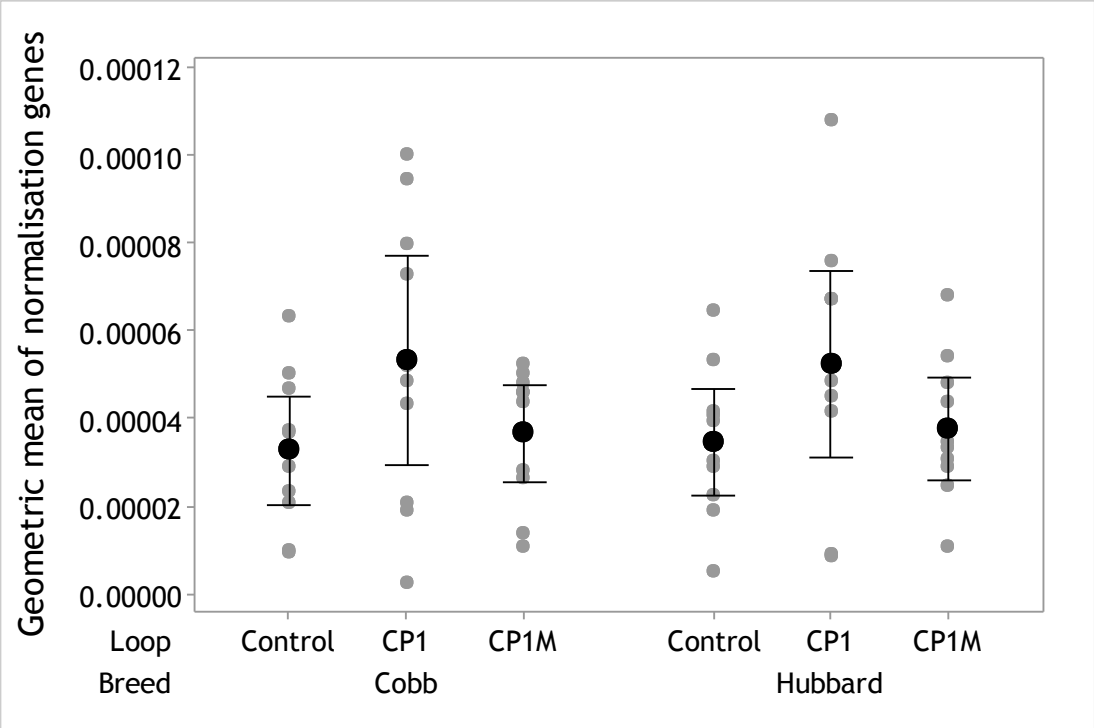

Figure 4.8. Analysis of normalisation genes

The geometric mean data was analysed by ANOVA which indicated a significant increase in the genes used for normalisation in the loops containing CP1.

4.3.2.6. Gene expression analysis: mRNA expression of any of the genes measured here was not affected by the broiler breed following exposure to *C. perfringens*. Expression of various target genes was affected by the *C. perfringens* isolates used in this experiment (Table 4.3). *IL-17F* mRNA expression was increased following infusion of the wild type CP1 isolate (p=0.034) in comparison to the control. On the contrary, infusion with the avirulent CP1ΔnetB isolate resulted in increased expression of *IL-1β*, *CXCLi1*, *IRAK*-4 and *B-LA* when compared to chambers infused with the control preparation or the wild type CP1 treated loops. *IFN-γ* mRNA expression was increased in chambers infused with wild type and mutant bacteria (p=0.025), whereas *CXCLi2* and *FAS* mRNA expression was reduced in the presence of wild type and mutant bacteria in comparison to the control treatment (p=0.0001 and p<0.0001respectively). CP1 presence reduced *IL-10*, *MUC2* and *MUC13* mRNA gene expression compared with the control and CP1ΔnetB (p=<0.0001, p=0.001 and p=0.025). Expression of *IL-17A* and *MUC5* mRNA was detected at similar levels across all three loop treatments.

Table 4.3. Mean log10 qPCR data for loops infused with *C. perfringens* culture with and without NetB

Breed	Loop Treatment	IL-6	IFNg	IL-1b	CXCLi1	CXCLi2	IL-17A	IL-17F	IL-10	IRAK4	Fas	BLA	Muc2	Muc5ac	Muc13
Cobb	Control	-0.91	-1.71	1.58	-1.36	1.57	-1.22	-2.97	-0.29	-2.48	-0.96	6.9	2	-1.37	-1.13
	CP1	-1.16	-0.97	1.37	-1.28	1.12	-1.2	-2.59	-0.87	-2.5	-1.58	6.85	1.8	-1.4	-1.19
	CP1ΔnetB	-0.8	-1.33	1.92	-0.92	1.12	-1.33	-2.81	-0.49	-2.2	-1.28	12.84	2.08	-1.57	-1.01
Hubbard	Control	-1.22	-1.98	1.39	-1.6	1.43	-1.34	-3.01	-0.59	-2.42	-0.97	8.82	2.01	-1.44	-0.16
	CP1	-1.32	-1.21	1.09	-1.47	0.83	-1.59	-2.7	-1.25	-2.49	-1.62	6.75	1.54	-1.72	-0.44
	CP1ΔnetB	-0.93	-1.39	1.71	-1.23	0.95	-1.5	-2.94	-0.56	-2.2	-1.21	16.2	1.99	-1.69	-0.29
P value*	**Breed**	0.29	0.292	0.16	0.057	0.09	0.124	0.396	0.064	0.882	0.903	0.434	0.38	0.072	0.181
	Loop treatment	0.024	<0.0001	0.002	0.026	0.0001	0.432	0.034	<0.0001	0.0004	<.0001	0.0001	0.001	0.146	0.025

Data are presented as the mean of log10 transformed data for each loop treatment within the two commercial breeds. There were no significant interactions in the mRNA expression data as part of this study.

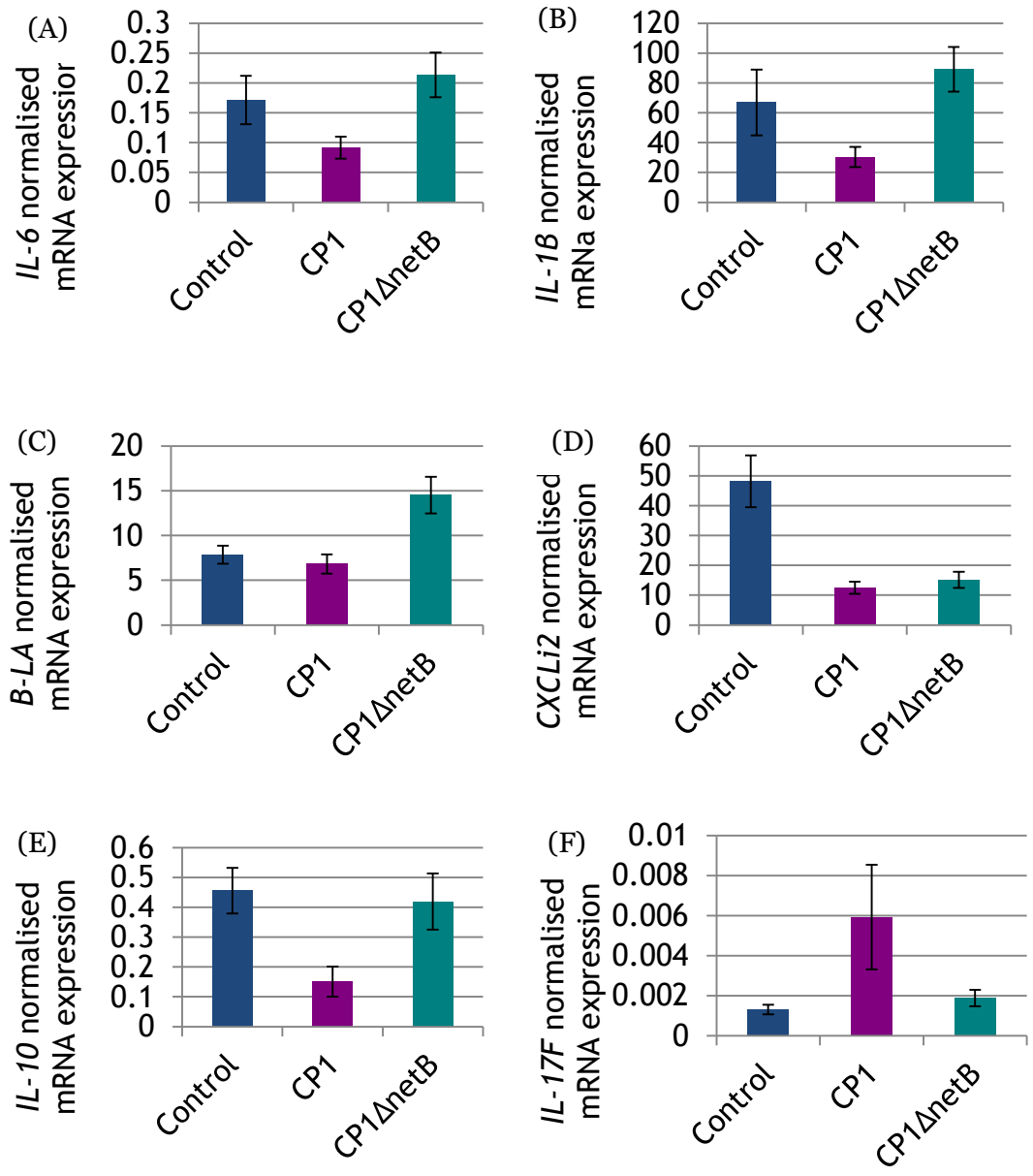

Figure 4.9. mRNA expression of genes related to host responses

IL-6 (A), IL-1β (B), B-LA (C), CXCLi2 (D), IL-10 (E), IL-17F (F) expression post infusion of bacteria + culture supernatant in broiler duodenal loops. No difference was detected between Cobb and Hubbard broilers.

4.4. Discussion

Our results show that specific cell populations associated with antigen detection are significantly different in the duodenum of broilers which have both been selected for growth traits over previous decades. Here, the *C. perfringens* isolates used only differ in their ability to produce the NetB toxin meaning any differences between loop treatments is likely to be related to the presence or absence of this toxin. We confirmed previous results that the presence of NetB affects gene expression as early as 4h post infusion in the duodenum of broilers. Cobb and Hubbard broilers have been reported to have differing responses during experimental NE (Jang et al., 2013). After experimental challenge with *Eimeria* and *C. perfringens*, Cobb broilers presented with the highest lesion scores and the largest reduction in weight gain compared with Hubbard and Ross broiler breeds. Infected Cobb broilers also had increased NetB antibody levels compared with their commercial counterparts. These results indicate that some commercial lines may be more resilient and resistant during NE infections (Jang et al., 2013). Our results have indicated little difference between Cobb and Hubbard in the first hours post exposure but the increased immune cell populations in the Hubbard breed may contribute to the improved adaptive response measured in this previous work.

We showed that staining for KUL01 and TCR1 cells highlighted a greater positive area for both markers in Hubbard broilers compared with the Cobb line. The anti-KUL01 antibody labels both macrophages and dendritic cells in the chicken (Mast et al., 1998). Increased numbers of these cell types in the

Hubbard broilers may indicate improved intestinal surveillance of pathogens. These cell types readily detect molecular patterns from pathogens, phagocytose antigens and follow up with signals to attract other cells if required. Increased KUL01 positive cells may contribute to the improved response detected in Hubbard broilers compared with Cobb in the studies reported by Jang et al., 2013. In addition to the breed effect, here Cobb broilers also had reduced KUL01+ percentage area in CP1ΔnetB infused loops compared to control or CP1 wild type and the same loops in the Hubbard broilers. Despite this cell marker being reduced, there was no simultaneous interactions between the broiler breed and loop treatment in any of the target genes measured. Further work may elucidate whether cells from these two broiler breeds differ in their ability to respond after exposure to *C. perfringens*. Transgenic reporter chickens with fluorescent macrophages were recently developed (Balic et al., 2014). These birds have been used to investigate the behaviour of the macrophage population throughout embryonic development and to visualise lymphoid structures in older birds. New technologies such as these could provide insight into the interactions between pathogens, like *C. perfringens* at the site of infection and whether they migrate after activation to influence other immune cells.

TCR1 is the antibody marker for γδ T cells in the chicken (Sowder et al., 1988). γδ T cells make up part of the intraepithelial lymphocyte (IEL) population and they are also found in the lamina propria (LP). Cells in these two locations have different roles to play during innate responses with IELs producing IFN-γ and those in the LP producing IL-17 (Vantourout and

Hayday, 2013). These cells can be activated via T cell receptor dependent and independent methods. This can occur through interactions with the MHC antigen presentation complex or γδ T cell direct interaction with microbial and host derived compounds (Bennett et al., 2015). Although this type of cell is not known to have a role against *C. perfringens* during the host response, they are known to play an important role in protection against *Eimeria* parasites in the chicken (Choi and Lillehoj, 2000; Rothwell et al., 1995; Vervelde et al., 1996). γδ T cell numbers increase in the IEL during *Eimeria* infections (Choi and Lillehoj, 2000). *Eimeria* sporozoites are often next to or within TCR1+ cells in immune birds compared with naïve birds indicating this cell type is important for protection (Vervelde et al., 1996). It is possible that the increased immune cell population detected in Hubbard broilers in our experiment plays a key role in the protective responses measured by Jang et al., 2013 as *Eimeria* is used as a pre-disposing factor in the experimental challenge. The short duration of the current experiment made investigating changes in body weight, gross lesion scores and antibody levels unfeasible to compare with the previous study. Despite Hubbard broilers having an increased positive area of γδ T cells when compared with the Cobb broilers, there was no difference in the levels of *IL-17A*, *IL-17F* or *IFN-γ* mRNA expressed across the two breeds. Both *IL-17A* and *IL-17F* induce antibacterial responses and their expression levels were increased during *C. perfringens*-*Eimeria maxima* co-infection (Lee et al., 2013). Currently, it is unknown whether the immune cells present in Cobb broilers have an increased activity to compensate for the lower number of cells. It should be noted that at the age of surgery the two broiler breeds used had significantly different body

weights with the Cobb being heavier than the Hubbard. Intraepithelial lymphocytes increase in the first 8 weeks of life in white leghorn chickens kept in specific pathogen free conditions and then diminish at 18 months (Vervelde and Jeurissen, 1993). Further work is required to determine whether Cobb birds have lower numbers of immune cells over these few weeks. Little evidence exists comparing the immune systems of commercial broilers within one study. It may be possible that body weight impacts on intestinal immunity. Weight matched Hubbard broilers rather than age matched broilers may have a similar number of cells. An experimental challenge investigating changes in the immune cell populations and the mediators they produce over time may provide understanding as to why some commercial broilers thrive better under challenge conditions than others.

Here, *IL-17A* was similarly expressed between all loop treatments but *IL-17F* was increased in loops where NetB was present. This previous work detected an increase two days post infection. We may be detecting an earlier response of *IL-17F* to *C. perfringens* which has not yet been measured so soon after exposure. The increase in *IL-17F* mRNA in the presence of NetB could indicate a role for innate γδ T cells in NE infections but further investigation is required to determine if increased numbers of these cells contributes to protection in NE. It is possible that they play a role later in infections.

A number of genes measured here are reduced or unchanged (in comparison to the control) in loops containing NetB when we would have expected increased mRNA levels. *IL-1β*, *IRAK-4* mRNA expression and antigen presenting *B-LA* mRNA expression in loops where NetB was present was

similar to that detected in the control loops. Loops without NetB had increased mRNA expression of these three genes which have roles in inflammation, antigen detection and antigen presentation (Cheng et al., 2011; Li et al., 2002; Salomonsen et al., 2003). During early responses it was expected that these genes would be increased in the presence of virulent bacteria but two *in situ* loop experiments using virulent isolates have determined the expression of these three genes to be either similar or reduced to levels detected in control loops (Chapter 3). NetB has 30% sequence similarity to, the pore forming toxin, α-Hemolysin from *Staphylococcus aureus*. This toxin is involved in evasion of *S. aureus* by altering the innate cytokine responses which in turn prevent immune cell influx to the site of infection (Tkaczyk et al., 2013). *S. aureus* α-Hemolysin also contributes to evasion of the host immune system by mediating the killing of perivascular macrophages which in turn prevents the up-regulation of chemokines required for neutrophil recruitment and pathogen clearance (Abtin et al., 2014). NetB may also have similar effects to induce evasion.

Other genes also measured here contribute to our theory of *C. perfringens* immune evasion. *CXCLi1* and *CXCLi2* are chemokines which have a similar amino acid sequence identity and bind with the same receptor, CXCR1. *CXCLi1* attracts heterophils to the site of infection (Poh et al., 2008). Heterophils are phagocytic and are generally one of the first responding cells against infective pathogens (Genovese et al., 2013). Chicken peripheral blood monocytes increase *CXCLi1* mRNA soon after two hours exposure to *C. perfringens* alpha-toxin but similar studies have not been carried out in the

presence of NetB for comparison to the current work (Sumners et al., 2012). Heterophils were present in the duodenum at similar numbers across all loop treatments but *CXCLi1* expression was higher in loops where NetB was absent. This could be another contribution of NetB towards *C. perfringens* avoidance of host immune cells. On the other hand *CXCLi2* was reduced in the loops that were infused with either bacterial isolate in comparison to the control. *CXCLi2* is mainly chemotactic for monocytes to sites of infection. This may be an evasion strategy of all *C. perfringens* bacteria to avoid uptake by migrating immune cells which is mediated by a component other than NetB. This response may diminish later in exposure as increased *CXCLi2* was detected in the ileum in the days after experimental infection with *C. perfringens* (Lu et al., 2009). Further investigation is required to determine whether *C. perfringens* utilises NetB in similar way to α-Hemolysin by *S. aureus* to avoid host immune responses and how other components may also contribute to this. The development of *in vitro* immune cell cultures may be able to provide insight into *C. perfringens* innate cell evasion in the presence and absence of NetB.

Antigen presenting cells are an important source of *IL-10*, the anti-inflammatory cytokine. Some pathogens influence increased expression of this mediator; preventing activation of other immune cells. This permits evasion of the host immune system and therefore promotes survival of the pathogen (Iyer and Cheng, 2012). Duodenal loops containing NetB, in this study, showed reduced *IL-10* mRNA expression. This is in disagreement with other studies as *IL-10* was increased in experimental infection studies using a

co-infection model with *Eimeria* (Bangoura et al., 2014; Collier et al., 2008; Park et al., 2008). Also, birds infected with *C. perfringens* alone as part of the same experiments showed no differential expression of *IL-10* mRNA in comparison to uninfected controls (Collier et al., 2008; Park et al., 2008). An experimental NE model using only *C. perfringens* detected reduced *IL-10* seven days post infection (Cao et al., 2012). Together these studies indicate that any evasion strategy initiated by NetB is unlikely to be mediated via the up-regulation of *IL-10*.

As well as any possible evasion strategy our results showed that NetB presence may also disrupt barrier molecule expression. *MUC2* and *MUC13* mRNA expression was lower in loops where NetB was present compared to the other loop treatments. Mucins are the first barriers that bacterial pathogens come into contact with in the intestine (Kim and Khan, 2013). When co-challenged with *Eimeria* and *C. perfringens* broilers showed reduced mRNA expression of both these mucins in comparison to unchallenged controls or challenged birds treated with antibiotics in their feed (Forder et al., 2012). *MUC2* is a secreted molecule whilst *MUC13* is bound to the cell membrane (Maher et al., 2014). Reduced expression of these molecules could provide easier access for *C. perfringens* to reach the epithelial cell layer. As NetB causes disruption to epithelial and goblet cells, which are responsible for the production of these mucins, it is possible it contributes towards the reduced expression of the mucin genes.

Similar to the *CXCLi2* results mentioned previously, *IFN-γ* mRNA expression was influenced by both bacterial isolates used in this experiment. *IFN-γ*

mRNA expression was increased in comparison to the control loops indicating a component other than NetB induced this response. In previous studies with the same intestinal loop model (Chapter 2 and Chapter 3) we showed an increase in IFN-γ mRNA expression following exposure to culture supernatant alone and bacterial culture from two wild type isolates (CP4, CP5). The two wild type isolates used differed in their virulence profiles but induced similar IFN-γ mRNA expression. These results, in combination with the data from the current study indicate that the early IFN-γ response seen in all our experiment may be mediated by factors other than NetB from *C. perfringens*. It is possible that alpha-toxin mediates this response as IFN-γ was increased in peripheral blood mononuclear cells after two and four hours exposure to alpha-toxin with and without LPS in comparison to control cells (Sumners et al., 2012). A *C. perfringens* challenge model detected an increase in IFN-γ mRNA one week after infection indicating this cytokine may have a role throughout infections (Cao et al., 2012).

Despite there being key differences in the immune cell populations of these two commercially available broilers there does not appear to be widely altered innate immune responses between the Cobb and Hubbard birds. It is possible that adaptive response will play a more important role in defence against *C. perfringens*. The difference in cell numbers could have a greater effect on the expression of immune mediators in the days/weeks after infection, rather than in the initial hours, which could sway the adaptive T helper cell and B-cell responses towards a protective response.

The presence of NetB toxin appears to dampen down pro-inflammatory responses with reduced IL-6 and IL-1β being detected. This could be an evasion strategy for *C. perfringens* to persist in the broiler intestine and similar results have been indicated in earlier chapters of this thesis. Further study is required to determine whether NetB sustains these mechanisms over a longer infection and contributes to the persistence of *C. perfringens* in the broiler. No clear mechanism for cell death has been established in this study and it is likely that as well as FAS, many other molecules will play a role. The development of *in vitro* assays for the chicken and the use of transgenic birds will further advance our knowledge of the interactions between this pathogen with host cells, such as macrophages and dendritic cells, and determine whether their activity alters in the presence of *C. perfringens* and its toxins. Further investigation of disease progression and the cell types involved in the response could highlight whether results from this intestinal loop model are relevant to typical progression of NE in the field.

Chapter 5 : Induction of sub-clinical necrotic enteritis with administration of *C. perfringens* and high dietary protein

5.1. Introduction

Legislation from the European Union prohibiting the use of in-feed antimicrobials and consumer demand for "antimicrobial free" broiler meat over the last decade has sparked a renewed interest in the research into production diseases, such as Necrotic enteritis (NE). With this interest a number of experimental infection models have been tested to investigate disease pathogenesis and host responses with the aim to develop disease preventative measures. In particular such models have been used to investigate host-pathogen interactions, bacterial isolate pathogenicity, vaccine treatments and therapeutic interventions for the disease (Antonissen et al., 2014; Jang et al., 2013; Jiang et al., 2009; Keyburn et al., 2010; Kulkarni et al., 2010; Kyung et al., 2012; Lanckriet et al., 2010; Lepp et al., 2013; Mikkelsen et al., 2009; Park et al., 2008; Parreira et al., 2012; Saleh et al., 2011; Xu et al., 2014).

Experimental infection models for NE are carried out in a number of ways. Often these utilise co-infections with *Eimeria* parasites to increase broiler susceptibility to *C. perfringens* and ensure the development of intestinal lesions (Jang et al., 2013). Co-infections with infectious bursal disease virus are also commonly used to encourage the development of disease (Lanckriet et al., 2010). Others use only high levels of *C. perfringens* administered over a few days to induce NE lesions (Jiang et al., 2009). Models using co-infections induce different responses in the host compared with models using only one infectious agent. For example, *Eimeria* infections cause different expression patterns of barrier molecules, such as mucins, when compared

with *C. perfringens* alone or together as co-infections (Collier et al., 2008; Forder et al., 2012). Kitessa et al, (2014) detected increased Mucin 13 after *E. acervulina* and *E. maxima* infection. Increased levels of mucins may encourage *C. perfringens* growth indicating that infection with other pathogens can in fact provide the correct conditions for NE to develop (Collier et al., 2008). Improved understanding of NE disease pathogenesis could elucidate the role of pre-disposing factors and why they do not consistently induce the disease.

Previous work in our laboratory and others has used the intestinal ligated loop models to investigate the role of bacterial toxins in disease (Athanasiadou et al., 2015; Caserta et al., 2011; Verherstraeten et al., 2013). In the absence of any avian mucosal cell line, this model provides a method of investigating early host responses soon after exposure to bacterial antigens; it also allows the use of fewer birds to investigate host pathogen interactions as more than one treatment can be applied per bird. It is unclear, however, how host responses as measured by immune cell populations and mRNA expression levels in these ligated loop models compare to natural infections in the field or to experimental infections. Early host responses are responsible for activating beneficial protective immunity; but the direct infusion of bacteria in the duodenum may activate responses in a different manner to field infections, where *C. perfringens* multiplies in response to other changes in the gut at the same time. Previous work at our facility used dietary changes and variations of *C. perfringens* administration to induce NE resulted in inconsistent experimental disease. More consistency was observed

when broilers were challenged alongside a number of pre-disposing factors. For example, the inclusion of fish meal in the diet, vaccination for infectious bursal disease virus on Day 16, an overdose of coccidial vaccine on Day 18 alongside multiple doses of *C. perfringens* (twice daily for four days) caused subclinical NE lesions in 19/56 challenged birds (Saleem, 2013). Although the loop model has many advantages, it also has some disadvantages, the main being the time window within which we can test host responses. To facilitate future studies and comparisons in host responses obtained with the loop model and experimental infections, we here aimed to trial various protocols to induce NE in a reproducible manner at our facility. *C. perfringens* along with an increase in dietary protein were tested with the aim of inducing NE without other influencing infections which could impact the structure and function of the intestine.

5.2. Methods

5.2.1. Broilers

One hundred and sixty eight Ross 308 male broiler chickens were obtained from PD Hook (Hatcheries) Ltd, Dalton, North Yorkshire and were housed in floor pens under conventional housing conditions. Food and water was provided ad libitum. Chicken starter feed, free of coccidiostats, was offered for the first 14 days of life (20% protein). On day 15 the diet was switched to a high protein diet (28%) for the remainder of the study to promote *C. perfringens* colonisation of the intestine.

5.2.2. Experimental design and Infection protocols

Four infection protocols obtained from the University of Guelph and Zoetis were evaluated and compared for their ability to induce NE lesions to control groups.

Table 5.1. Experimenal groups and infection protocols for induction of NE

Treatment Group	Control /infected	Method of admin	Start of infection	Duration of infection (days)	No. of doses/day	Days of Post mortem
T01	Control	In-feed	D15	3	2	D19, D20
T02	Control	Gavage	D15	3	2	D20,D21
T03	Infected	In-feed	D15	3	2	D20
T04	Infected	In-feed	D16	2	2	D19
T05	Infected	Gavage	D15	3	2	D20,D21
T06	Infected	Gavage	D15	3	1	D20,D21

Two of the infection protocols entailed administration of *C. perfringens* in-feed (T03 and T04), whereas the other two entailed gavaging of *C. perfringens* (T05 and T06). T01 was an in-feed control and T02 was a gavage control. T03 (Guelph) and T04 (Zoetis) were both in-feed infected and T05 (Guelph) and T06 (Guelph reduced) were gavage infected. T06 was included to reduce handling stress on the birds. The six treatment groups were allocated between the twenty four pens used (4 pens per treatment). Seven broilers were housed in each pen (2.25m^2) and the pens were split across two rooms (2 pens/treatment/room). Table 5.1 summarises the infection protocol for each group. For the first 15 days of the experiment, all birds were treated in the same manner; treatment application started on D15 and continued for

three days; with the exception of T04 which started on D16 and continued for two days. Groups that were infected via the feed underwent post-mortem on D19 and D20 whereas gavage infected birds underwent post mortem on D20 and D21 as per the original protocols.

5.2.3. Bacterial Culture of *C. perfringens* CP4

C. perfringens CP4 (University of Guelph, Canada), from frozen glycerol stocks was cultured on sheep blood agar plates overnight at 37°C anaerobically. Bacterial DNA was extracted using the boil prep method. Briefly, one colony from each plate was transferred to 100µl of RNase/DNase free water. This was boiled for 10mins and then centrifuged at 14000g for 10 minutes for DNA extraction. The supernatant was removed and used as the sample for each isolate. Phire hot start II DNA polymerase (Finnzymes, Thermo Scientific) was used in 20µl reactions. Each sample was checked for the presence of NetB to ensure isolate virulence. After confirmation of NetB, 5-7 colonies were sub-cultured into 5mls TPG broth (5% tryptone, 0.5% protease peptone, 0.4% glucose, 0.1% thioglycolic acid) and kept overnight at 37°C anaerobically. This culture was then stored anaerobically at room temperature.

Two days prior to each challenge day an increased volume of culture was prepared by transferring 3% (v/v) CP4 infected TPG to sterile TPG broth and kept anaerobically overnight at 37°C. The evening before each challenge, the appropriate volume of inoculum for infections was prepared. Again, 3% CP4 infected TPG (prepared the previous day) was transferred to sterile TPG and kept overnight at 37°C anaerobically. An aliquot of culture was kept for

enumeration of *C. perfringens* and again to determine the presence of NetB after culturing. 100µl of each culture was diluted 1:10 with RNase/DNase free water. The same protocol for DNA extraction was performed as before.

5.2.4. NE Challenge

In-feed infection groups (T03 and T04) were offered 5x10^8 CFU of *C. perfringens* culture mixed with the high protein diet (28% protein) at a ratio of 1:1 twice daily. 45mls of culture with 45g of feed per bird was prepared and offered morning (approx. 8am) and afternoon (12pm). Gavage infection groups (T05 and T06) were administered 2mls of 5x10^8 CFU *C. perfringens* culture as indicated by the experimental timetable (Table 5.1). Control groups received, sterile bacterial culture TPG media, either mixed with feed (T01) or via gavage (T02) in the same manner as the infection groups. Group T03 and T04 underwent feed withdrawal for 16 or 2 hours, respectively, prior to the initial placement of *C. perfringens* as per respective protocols.

5.2.5. Body weight and lesion score

Individual body weights (BW) were recorded on D0, D7, D14 and prior to euthanasia to estimate growth performance over the study. This created three growth periods: D0-7, D8-14 and D15-Final. The average daily weight gain (ADWG) was calculated for each growth period as:

$$\frac{BW - Initial\ BW}{No.of\ days\ in\ growth\ period}$$

Birds were euthanized with pentobarbital on the days indicated in Table 5.1. Following euthanasia birds were opened and the gastrointestinal tract was

removed and inspected from the outside. A sample was removed from the bottom of the duodenal loop and fixed in 10% formalin. The G.I. tract was then opened to evaluate the severity of NE lesions throughout the small intestine which were assessed on the scale published by Keyburn et al., 2008.

5.2.6. Histological examination

Formalin fixed duodenal tissue from 3 birds/pen, chosen at random, were embedded in paraffin. Samples from NE lesions were also processed. Sections were cut and stained with haematoxylin and eosin (H&E) and were scored for microscopic histopathological lesions in a scale from 1 to 3 as used in previous chapters; Score 1 indicated that there was no pathology observed. Score 2 indicated villi fusion in areas of the section and cell death down to the crypts but no crypt loss. Score 3 indicated villi fusion throughout the section and areas of complete crypt loss. One section per bird was evaluated.

5.2.7. Heterophil quantification

The H&E sections used in the histological examination were also examined under x400 magnification for the quantification of heterophils. Five high powered fields were chosen throughout the section and the number of heterophils counted in each, to calculate the average number of heterophils across these fields of view.

5.2.8. Statistical Analysis

Growth performance parameters (BW, ADWG) were analysed with one way ANOVA across all six treatment groups. Heterophil numbers were analysed with one way ANOVA, within each method of infection (in-feed or gavage).

Due to the skewed nature of the heterophil counts, the data were Log10 transformed prior to analysis. The appropriate blocks were included in the statistical model for the spatial areas of the treatment groups. The blocks were pen, area (for the spatial block within each room) and room. Macroscopic and microscopic intestinal lesion scores were analysed using the Kruskal-Wallis test comparing treatment groups. Genstat (16th Edition) was used for all analysis.

5.3 Results

5.3.1. Presence of NetB

All plates used for initial culture of CP4 were confirmed as NetB+. This gene was also present in all of the cultures used on the three days of infection as confirmed by PCR.

5.3.2. Growth performance

There was no significant effect of the treatment on the BW of broilers. During the last growth period (D15-Final) ADWG for the in-feed infected groups (T03=53.57g/d, T04=58.68g/d) was similar to that of the in-feed control (T01=53.52g/d). Similarly, there was no difference between the gavage control (T02=57.26g/d) and the gavage infected (T05=60.49g/d, T06=60.79g/d) birds. The ADWG showed a tendency to be significant for T01 at D0-7 (p=0.052). Broilers in T01 increased their ADWG the least over this initial growing period (T01=15.14g/d) compared with the other treatment groups (T02=16.14g/d, T03=16.43g/d, T04=15.82g/d, T05=16.86g/d,

T06=17.07g/d). The ADWG of T01 was similar to that of the other groups for the other two time periods; D8-14 and D15-Final weight. (Table 5.2)

5.3.3. Lesion Scores

At post mortem three broilers were positive for gross NE lesions; all positive birds originated from infected groups. One bird originated from T04 (Zoetis, in-feed infected); this had a few areas of focal necrosis and was classed as Score 2 (Figure 1A). The other two birds presented larger areas of necrosis and were classed as Score 5 (T06, reduced gavage) and 6 (T03, Guelph in-feed) (Figure 1B and C). The majority of broilers scored zero with no gross lesions visible. 20 from the 168 broilers lesion scored, presented with a superficial, removable fibrin and were assigned score 1. These were distributed throughout the six treatment groups, including controls, but were more common in T04 (in-feed), T05 and T06 (gavage and gavage reduced) (5/28 in each treatment group) compared with the two control groups and T03, in-feed infected. Figure 5.2 shows the distribution of macroscopic lesion scores. There was no significant difference between treatments (p=0.166).

Table 5.2. Growth performance and birds positive for NE lesions

	Treatment Group							
	Control Groups		Infected Groups					
Average weight/bird (g)	**T01**	**T02**	**T03**	**T04**	**T05**	**T06**	**SEM**	**P value**
D0	39.3	39.4	39.4	40.2	40.1	39.4	0.7	0.601
D7	145.3	152.3	154.5	150.9	158.1	158.9	4.6	0.078
D14	404.5	426.8	438.4	429.4	431.8	435.8	16.4	0.392
Final	699.4	797.5	759.9	707	815.4	808.3	26.77	0.246
Average Daily Weight gain (g)								
D0-7	15.14	16.14	16.43	15.82	16.86	17.07	0.609	0.052
D8-14	37.05	39.16	40.6	37.44	39.12	39.58	2.793	0.796
D15-Final	53.52	57.26	53.57	58.68	60.49	60.79	3.24	0.132
Birds Positive for NE								
	0/28	0/28	1/28	1/28	0/28	1/28	NA	NS

Mean body weights are presented for each treatment group. The results of ANOVA are presented with <0.05 deemed as being significant.

Figure 5.1. Lesions in broiler intestines

Lesions were scored based on the Keyburn scoring system which was reviewed and proposed for international adoption (Shojadoost et al., 2012). Lesion score 2 (A), score 5 (B) and score 6 (C) as detected in broilers from this study.

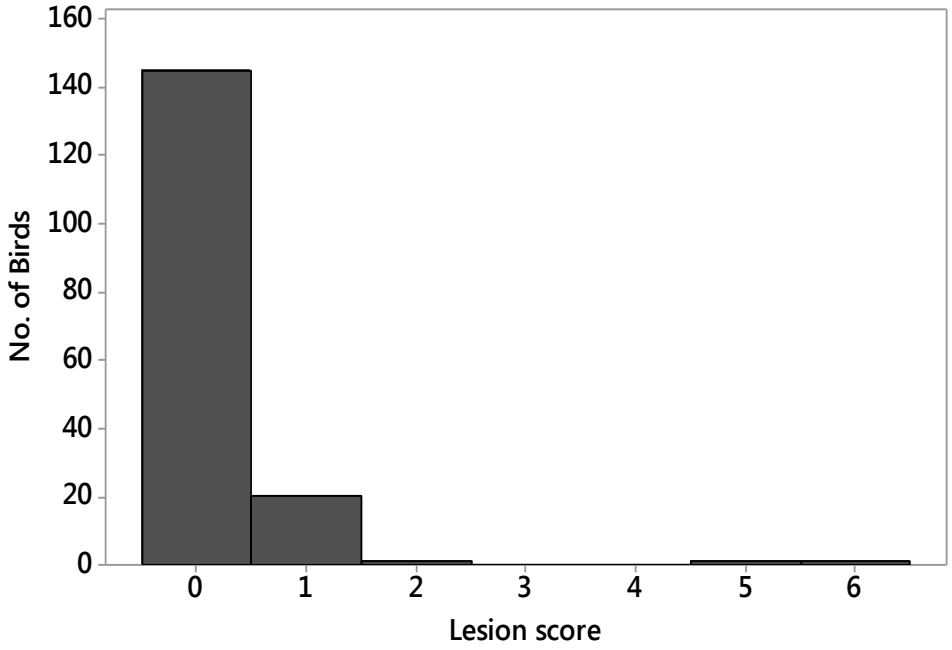

Figure 5.2. Histogram of Lesion scores

Histogram of macroscopic lesion scores from all birds at post mortem indicating the majority of birds were classes as score zero.

5.3.4. Microscopic Histological examination

Histopathological lesions typical of NE were detected only in one of the sections evaluated (Score 3). This was present in the broiler which had macroscopic lesion score 6 with NE lesions present throughout the small intestine. All other sections showed no indication of morphological damage (Score 1) therefore there was no significant difference between treatment groups ($p=0.517$).

5.3.5. Heterophil Quantification

Heterophils were detected in the majority of sections with only four sections having zero heterophils in the five high-powered fields of view analysed. Although this cell type was present in the majority of sections very few were seen on average. Neither T03 nor T04 had a difference in heterophil numbers from the in-feed control, T01 (Figure 2A). There was a difference between the in-feed infected broilers euthanized on D19 and D20, however. T04 birds euthanized as per protocol on D19 had more heterophils than T03 birds euthanized on D20 ($p=0.015$). Birds in the reduced gavage protocol, T06, had similar numbers of heterophils to the control gavage group, T02. The Guelph gavage group had significantly fewer heterophils than either of the other gavage treatment groups ($p=0.035$) (Figure 2B).

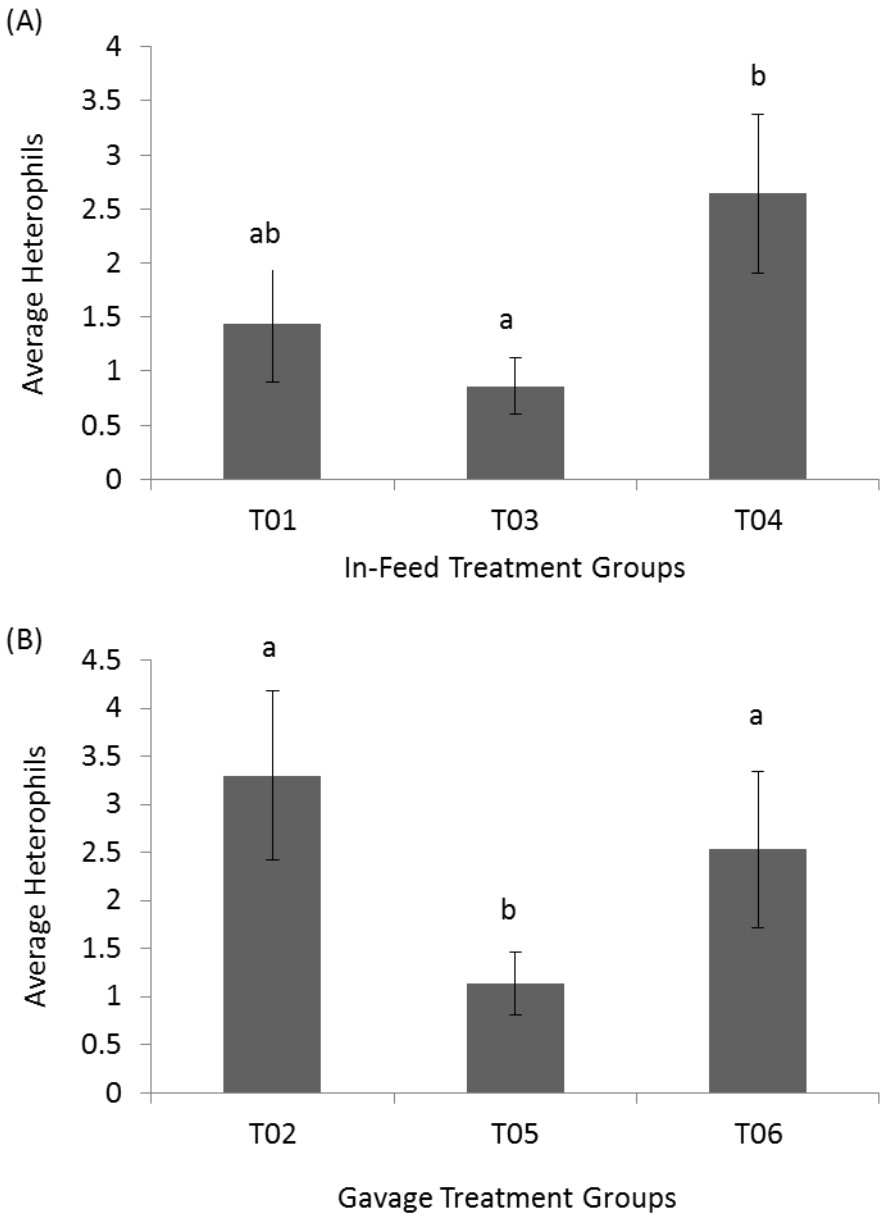

Figure 5.3. Average Heterophil counts in the duodenum of *C. perfringens* challenged boilers

Heterophils were counted in five high-powered fields of view in H&E stained sections. (A) In-feed treatments groups with different small case letters are statistically different ($p<0.05$). (B) Gavage treatment groups with different small case letters are statistically different ($p<0.05$).

5.4. Discussion

Previous work from *in-situ* loop experiments has detected differences in duodenal immune cell populations and changes in pro-inflammatory cytokine mRNA expression as well as other genes related to immune cell activity. Some responses measured in the *in-situ* experiments provided evidence for bacterial evasion of the host immune response and further characterisation over time is required to determine whether *C. perfringens* does in fact evade the broiler immune system. Here we aimed to develop an NE infection model which could be used to further investigate these responses over longer time periods.

The protocols tested here did not consistently induce NE in our broiler population, despite the fact that they are successfully used elsewhere to induce NE. In all treatment groups lesion scores were low and infection with *C. perfringens* did not impact on performance. Birds in all treatment groups had similar body weights to each other at the three time points monitored and had similar average daily weight gains (ADWG) indicating there was no effect of the experimental protocol on broiler growth rates during this experiment. Broilers allocated to the in-feed protocols gained weight similarly to those in the gavage protocol groups indicating feed mixed with liquid did not prohibit growth. Successful NE induction would likely have stalled broiler growth during the infection period (Kaldhusdal and Hofshagen, 1992).

The previously established protocols utilised a turkey starter diet with 28% crude protein (Kulkarni et al., 2010). Diet, in particular protein type and level

of inclusion, has been associated with NE pathogenesis. Increased fish meal, potato-based proteins and protein sources with high levels of trypsin inhibitors have increased *C. perfringens* counts in the digesta and increased the incidence of NE lesion scores (Drew et al., 2004; Palliyeguru et al., 2011, 2010). The form of the diet may impact on broiler host responses and *C. perfringens* numbers in the caeca of broilers (Engberg et al., 2002; Liu et al., 2006). For the present study both the starter and high protein diets were formulated in mash form which should have promoted *C. perfringens* growth and NE lesions but it appears other pre-disposing factors may be required to induce consistent experimental infection.

Recently, stocking density was implicated as a pre-disposing factor for NE in broilers (Tsiouris et al., 2015). Stocking density has an effect on broiler welfare and health etc. Birds in high stocking density situations (0.063m^2/bird) have increased levels of stress biomarkers , indicating that the welfare of these broilers may be compromised, even though there is little effect of this level of stocking density on production parameters (Najafi et al., 2015). Higher stocking densities also alter the morphology of in the intestine, reducing villus height which can contribute to reduced feed efficiency (Shakeri et al., 2014). Standard stocking density for countries in the European Union is 33kg/m^2 or the equivalent of each bird having 0.1m^2 (The Council of the European Union, 2007). Many recent studies investigating the effects of stocking density on broiler health and welfare define this as their low density groups with high density groups being reduced to approximately 0.065m^2/bird (Guardia et al., 2008; Houshmand et al., 2012; Najafi et al.,

2015; Shakeri et al., 2014; Sun et al., 2012). The effect of stocking density on NE has been previously tested using a co-infection model with *Eimeria* and *C. perfringens*. Although NE lesions were detected in both of the infected groups (Tsiouris et al., 2015) broilers in the higher density group (0.032m2/bird) showed increased gross lesion scores compared with the lower density group (0.067m2/bird). Low stocking densities may have been responsible for the lack of NE lessions in our experiment. Broilers here had a space allocation of 0.32m^2/bird giving them approximately 10 times more space than those in the high density group used by Tsiouris et al., 2015. It is possible that broilers were not stocked densely enough to promote the disease. Fewer birds per square meter also meant that litter quality was high throughout the duration of the experiment ensuring broilers were not exposed to wet litter which can be a predisposing factor to NE as well as effect of the disease (Hermans and Morgan, 2007; Hermans et al., 2006; Timbermont et al., 2011; Wilson et al., 2005). Future work should investigate increasing the stocking density with these protocols to induce NE. Increased stocking density has the ability to alter the composition of the broiler microbiota (Guardia et al., 2008). Changes in the microbiota populations may provide additional opportunities for *C. perfringens* to proliferate and cause NE.

Microscopic evaluation of haematoxylin and eosin stained slides indicated there was no difference between any of the groups in this study which was consistent with the gross pathology scores. Heterophils are often one of the first responding cells to bacterial infections in birds (Genovese et al., 2013).

Here, we saw very few of these cells in sections from the duodenum of broilers in this study but there are some statistical differences between treatment groups. The in-feed infected group, T03, showed different heterophil numbers from the T04 group, although neither of these groups differed from the in-feed control. The difference in heterophil numbers observed between these two groups may be attributed to the infection and/or euthanasia timing in relation to the infection, with T04 being euthanized a day earlier than T03. The reduced gavage group, T06 had a similar number of heterophils to the control gavage group, T02. The Guelph gavage group had fewer heterophils present in their duodenum. Stress can also affect the presence of heterophils in the intestine as well as infection (Calefi et al., 2014; Quinteiro-Filho et al., 2012). Birds in both the control (T02) and Guelph (T05) were handled in a similar manner throughout the duration of the experiment; both underwent gavage treatment twice daily for three days. It seems unlikely that the heterophil response is related to any stress response to the infection protocol.

In this study we were unable to consistently reproduce NE with these protocols as evaluated by growth, gross lesion scores and microscopic lesion scores. In the field, it is likely that broilers experience a number of predisposing factors which were not included in this experimental challenge, including *Eimeria* challenge and environmental stressors. An alternative area for future investigation could be to utilise more *in vitro* techniques to determine the effect of *C. perfringens* on host epithelial cells in the presence or absence of other infections and virulence factors. The development of

intestinal epithelial cell cultures as well as immune cell cultures could provide answers as to how *C. perfringens* initially interacts with chicken cells and whether this promotes the activation of virulence factors from the bacteria. Improving knowledge on NE disease pathogenesis could advance experimental challenge models and promote standardisation across the research community in turn helping to characterise the host response during early and later stages of the disease.

Chapter 6 : General discussion

6.1. General discussion

The research described in this thesis aimed to investigate innate immune responses to *C. perfringens,* and key virulence factors produced during necrotic enteritis infections, in broilers with the objective of developing a greater understanding of early host pathogen interactions and disease pathogenesis. This work predominantly utilised an *in situ* intestinal loop model to:

i) Investigate temporal patterns of broiler intestinal responses to culture supernatant positive for *C. perfringens* virulence factors in comparison to control (Chapter 2).

ii) Determine the intestinal response to culture supernatant produced from isolates differing in their virulence in the presence or absence of bacteria (Chapter 3).

iii) Examine differences in the early responses of two commercial breeds in the presence or absence of NE associated virulence factor, NetB (Chapter 4).

In the final experiment the aim was twofold: i) to experimentally induce NE using different infection protocols and ii) to establish a relationship in avian host responses between *in situ* and *in vivo* experiments (Chapter 5).

6.2. The use of impedance based signal to determine cytotoxicity of *C. perfringens* culture supernatants

Five different culture supernatants were prepared from *C. perfringens* isolates throughout this work. The first was produced from a co-culture of

two wild-type isolates taken from cases of NE, MPRL 4733 and 4739 (Chapter 2). In Chapter 3 isolates CP4 and CP5 were used. These two had previously been characterised to be virulent and avirulent, respectively, in experimental disease challenge models (Thompson et al., 2006). CP1 and its NetB null mutant, CP1ΔnetB, were used for the final loop study of this thesis (Parreira et al., 2012). The cytotoxicity of each culture supernatant was confirmed using xCelligence RTCA technology. Previously, lactate dehydrogenase assays (LDH) have been used to investigate *C. perfringens* culture supernatant cytotoxicity to the chicken liver male hepatocyte (LMH) cell line (Cheung et al., 2010; Keyburn et al., 2008; Lanckriet et al., 2010). The LDH assay is a colorimetric assay which measures the release of this enzyme which occurs during cell death (Chan et al., 2013). This assay however, only provides a snapshot at a chosen time after the addition of culture supernatant. The RTCA assay used as part of this thesis is able to monitor cell death/growth at numerous timepoints after the addition of culture supernatant. To our knowledge this is the first time impedance technology has been used to investigate the cytotoxicity of *C. perfringens* culture supernatant.

The presence of NetB was confirmed in the culture supernatants produced from our SRUC isolates (4733 and 4739) and the isolates provided from The University of Guelph (CP4 and CP1 wild type). CP1ΔnetB and CP5 were confirmed as NetB negative as culture supernatant at a dilution of 1:16 or more did not have cytotoxic effects on LMH cells. Figure 6.1 presents a comparison of all culture supernatants used throughout this thesis. Virulent culture supernatants diluted 1:2 (4733 and 4739) or 1:5 (CP1 and CP4) are

cytotoxic to LMH cells soon after addition. After four hours almost all cells are dead. On the other hand, CP1ΔnetB and CP5 culture supernatants diluted 1:5 do not have the same cytotoxic effects.

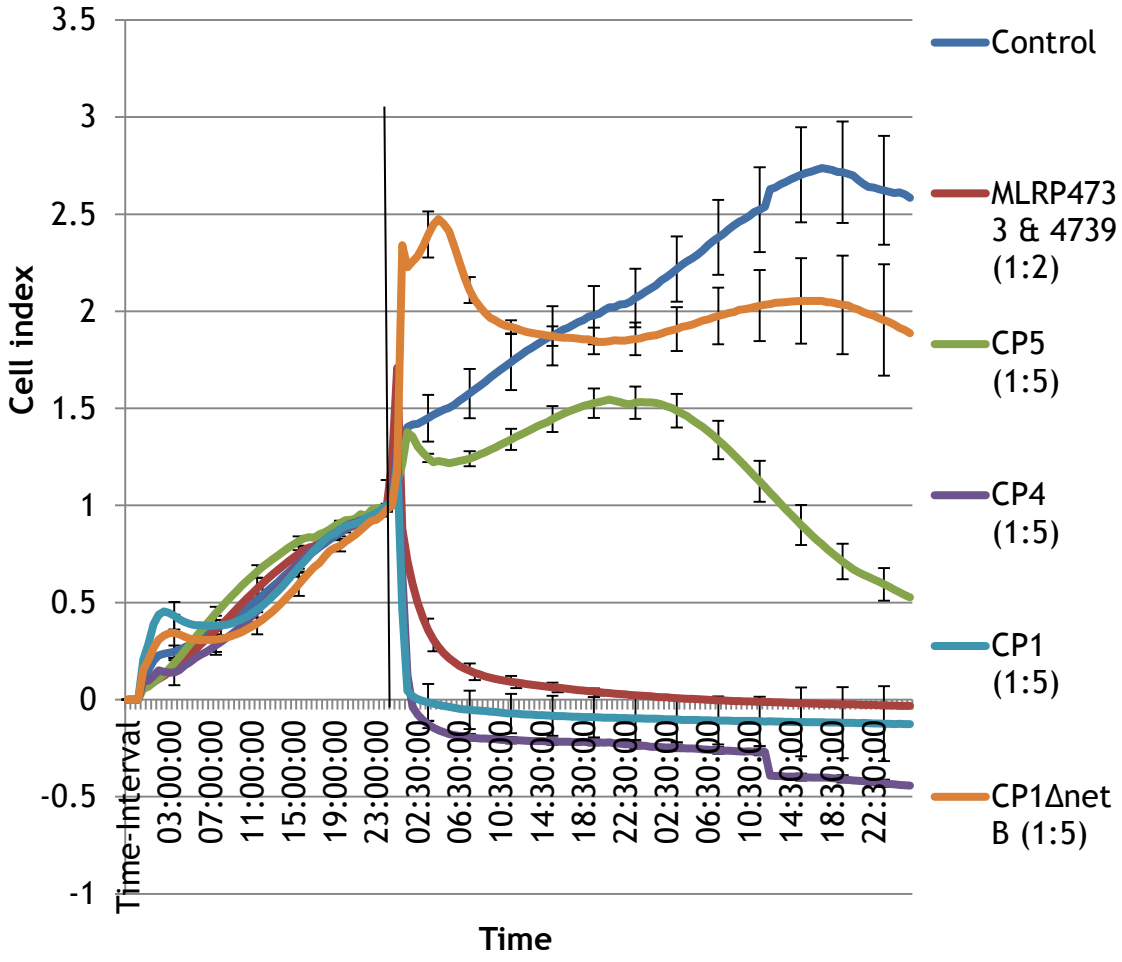

Figure 6.1. Comparison of all *C. perfringens* culture supernatants

Cytotoxicity of LMH cells incubated with *C. perfringens* culture supernatant from the different isolates used as part of this thesis. Cell index measured by RTCA xCelligence. LMH cells were cultured for 24 hour and culture supernatant was added (vertical back line). The numbers within brackets indicate the dilution factor for each supernatant.

It is possible that the culturing of both isolate 4733 and 4739 together may have lessened the cytotoxicity of the supernatant produced as opposed to single isolate cultures. The other two NetB+ isolates cultured alone induce cell death quicker than the co-culture used in Chapter 2. This is more evident in Figure 6.2 which shows further diluted culture supernatants. Culture supernatants from isolates CP1 and CP4 are highly cytotoxic at a dilution of 1:25, again inducing cell death substantially within the first four hours whereas, a 1:16 dilution of 4733 and 4739 supernatant did not have the same effect. Cell numbers were reduced in comparison to the control but not to the extent seen in cells incubated with CP1 and CP4. At this dilution there appears to be little difference between the two virulent isolates but at further dilutions CP4 remains cytotoxic when the effect of CP1 lessens. This may in part be down to the presence of TpeL in CP4 which is not present in CP1. All culture supernatants used in the *in situ* loop models were undiluted and likely played a part in mediating cell death within the intestine of broilers used throughout this work.

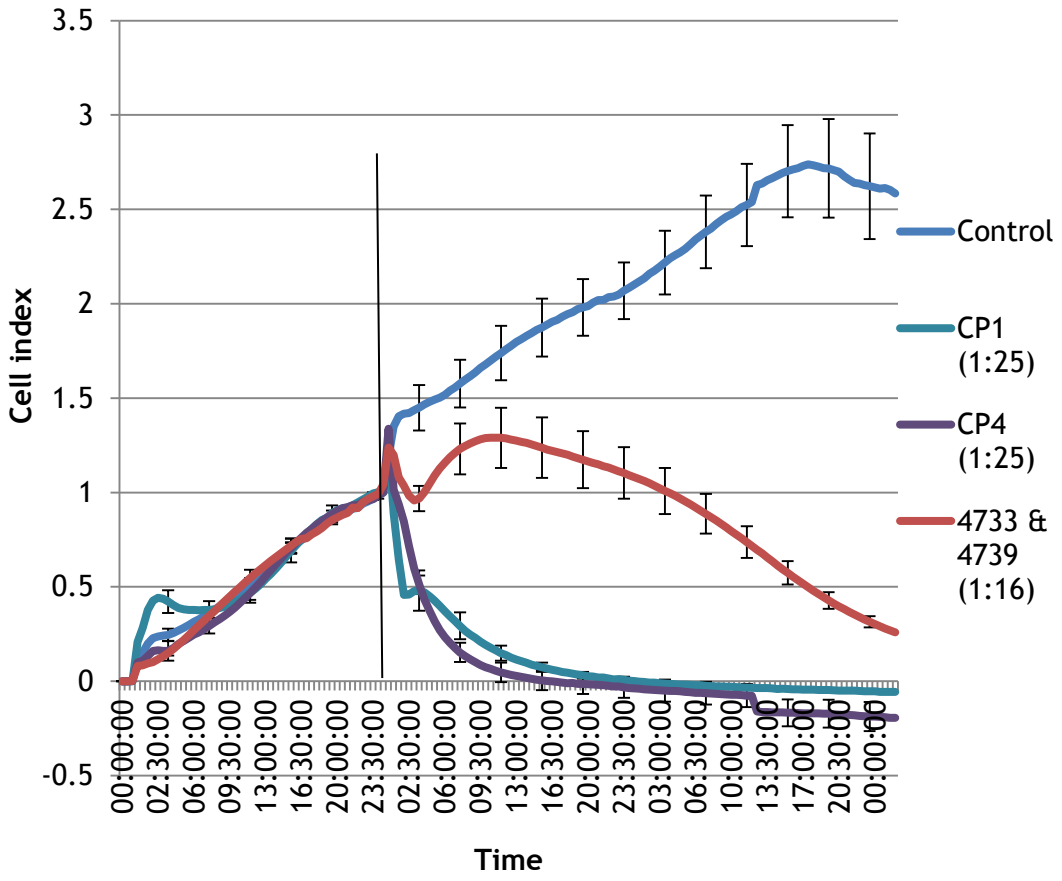

Figure 6.2. Comparison of virulent *C. perfringens* culture supernatants

Cytotoxicity of LMH cells incubated with virulent *C. perfringens* culture supernatant at higher dilutions. The numbers within brackets indicate the dilution factor for each supernatant.

6.3. Host responses to culture supernatant

Culture supernatants produced from *C. perfringens* cultures have been considered as vaccine candidates in a number of species including chicken, pigs and models of human infections (Berube and Wardenburg, 2013; Milach et al., 2012; Nagahama et al., 2015a; Salvarani et al., 2013). This is an approach that has been taken to protect broilers flocks against NE. Previous

studies have utilised culture supernatants, genetically altered toxins and formaldehyde inactivated toxins (toxoids) as vaccine candidates with the aim of reducing the incidence and severity of this disease (Fernandes da Costa et al., 2013; Kulkarni et al., 2007; Lanckriet et al., 2010; Lovland et al., 2004). Reduced lesion scores were seen in vaccinated birds after experimental challenge but there is little understanding behind the mechanisms of action for this protection. By investigating responses to culture supernatant over time and in comparison to bacterial cells we provide some insight into the host response to *C. perfringens* toxins.

Virulent culture supernatant used in Chapter 2 of this thesis tended to increase the mRNA expression of pro-inflammatory cytokines IFN-γ and IL-6 two hours post exposure but such responses were not observed when virulent or avirulent culture supernatants were used (Chapter 3). This may be down to a difference in components within the culture supernatants used between the two experiments. It is interesting to note that there was little difference between the avirulent and virulent isolates used in Chapter 3 in terms of the mRNA expression measured. Further investigation of the host response to culture supernatants may provide information on how these influence disease pathogenesis. There is still much to be understood about the release of virulence factors by *C. perfringens*. *In vitro* cultures produce NetB only at higher levels of bacterial growth and this is reliant on the virSR system (Cheung et al., 2010). This system senses the external environment and then responds as necessary. It was hypothesised that NetB is up-regulated in the chicken intestine once *C. perfringens* reaches a level of growth that activates

the virSR system. Interactions with host cells may also stimulate the up-regulation of toxin production by *C. perfringens* in the intestines of their host (Chen et al., 2013). *C. perfringens* sensory systems activated by Caco-2 human epithelial cell line stimulate the production of β-toxin and epsilon-toxin. It is unclear whether bacterial interaction with chicken intestinal epithelial cells could, in fact, cause up-regulation of *C. perfringens* virulence factors. In our experiment we included loops for bacterial transcriptomics for investigating changes in bacterial cells after they had come in contact with chicken intestinal cells. These up-coming results, from our collaborators, may indicate whether toxin genes are up-regulated soon after exposure to the intestine of the chicken.

Throughout our experiments, we used culture supernatant rather than purified toxins to gain an understanding of the host response to the variety of secreted factors produced by *C. perfringens*. It is possible that secreted components, other than toxins, may contribute to protective immunity as antibody responses and disease protection were seen in studies using some of these secreted proteins (Kulkarni et al., 2007, 2006). Despite the protection seen in these previous studies the response to the culture supernatant used in Chapter 2 and Chapter 3 was fairly muted. Our results in Chapter 3 indicate that pro-inflammatory mediators increased more when *C. perfringens* cells were present compared with the culture supernatant alone. The cells themselves, or components of the bacterial cell, may be useful as potential adjuvants to these and toxoid type vaccines.

6.4. Host responses to *C. perfringens*

One hypothesis in this project was that *C. perfringens* cells would activate the immune response differently to that of culture supernatant alone and that virulent isolates would induce different responses to avirulent isolates. The presence of bacterial cells in Chapter 3 resulted in increased pro-inflammatory cytokine (IL-6, IL-1β, IFN-γ) mRNA levels 0.5h after exposure but this effect was not maintained. Previous work investigating bird responses to *Salmonella enterica serovar Typhimurium* measured increased mRNA levels of various cytokines and chemokines in the first hours and days post infection (Withanage et al., 2004). Birds infected with this bacterium had increased CXCLi1, CXCLi2 and IL-1β mRNA expression levels compared to control birds in the ileum and cecal tonsils at six hours post infection. Wigley et al.,(2006) detected higher IL-1β, IL-6 and CXCLi1 in macrophage cultures after one hour exposure to *Salmonella* serovar Gallinarum or *Salmonella* serovar Typhimurium. We therefore expected the increase in these pro-inflammatory mediators to be sustained over the four hour period, especially in loops containing the virulent isolate.

One possibility for the reduced cytokine expression four hours post exposure was an evasion mechanism for *C. perfringens* to avoid immune cell influx to the site of infection. Two genes associated with macrophage/monocyte activity were differentially regulated in the presence of *C. perfringens* cells. Increased mRNA levels of the inhibitory gene NBL1 were detected whereas the MHC class II gene, B-LA, mRNA was reduced. Together the reduced cytokine and B-LA expression and increased NBL1 expression suggested that the function of macrophages and other cell types may be reduced during

infections with *C. perfringens* regardless of the virulence capabilities of the isolate.

As well as these results, IL-6 was reduced overall in birds that were administered the virulent isolate in comparison to the avirulent isolate. This highlighted that virulent isolates may have increased evasion capacities. As the two isolates used in Chapter 3 had very different virulence profiles it was not clear whether this reduced expression was related to the presence of NetB alone or other factors, such as TpeL, which was present on the CP4 isolate but not CP5.

The experiment described in Chapter 4 used the CP1ΔnetB mutant isolate. This meant that we could determine whether the reduced response in loops with the virulent CP4 isolate was the effect of NetB on host responses. The isolates used in this chapter were identical, other than the ability to produce NetB, and induced some similar responses. Both increased IFN-γ expression and reduced CXCLi2 expression. IFN-γ mRNA was also similarly expressed in loops containing either the avirulent or virulent isolate in Chapter 3. Together these results indicate that IFN-γ mRNA expression is not necessarily associated with *C. perfringens* virulence. It is likely to be mediated by a component of the bacterial cell or a secreted component from various *C. perfringens* isolates. Reduced CXCLi2 expression in loops with either CP1ΔnetB or CP1 wild type also indicates a response mediated via the bacterial cell or a component in the culture supernatant. This chemokine is also known as IL-8 and attracts monocytes from the blood (Barker et al., 1993; Poh et al., 2008). Again, the reduced expression of this chemokine

could have highlighted an evasion strategy for all *C. perfringens* isolates as both were able to elicit this response. Guo et al., 2015 showed *C. perfringens* and commercially available alpha-toxin increased expression of CXCLi2 in primary chicken embryo epithelial cells three hours post exposure. Although we did not detect an increase in CXCLi2 it is possible that the mRNA detected in our experiment was from epithelial cells and that *C. perfringens* cells and the alpha toxin produced by both isolates influenced the expression of this chemokine.

6.5. Host responses to NetB

Although CXCLi2 and IFN-γ were similarly expressed across loops containing the wild type and mutant bacteria, a number of genes were differentially regulated by CP1ΔnetB. Pro-inflammatory cytokines (IL-6, IL-1β, CXCLi1) were increased as well as genes related to immune cell activity (IRAK-4, B-LA). The same up-regulation was not detected for the wild type isolate. These results indicate that NetB may contribute to *C. perfringens* immune evasion in broilers. Immune evasion is commonly used by bacteria to prevent detection and promote survival within the host. There is some evidence that *C. perfringens* toxins contribute to immune evasion by promoting escape from the phagosome and therefore antigen presentation (O'Brien and Melville, 2004, 2000). The role NetB plays in immune evasion is unclear but pore forming toxins from other bacteria promote bacterial survival (Los et al., 2013). *Staphylococcus aureus* alpha-hemolysin promotes the uptake of bacterial cells into mast cells where they then avoid antimicrobial compounds produced by the mast cell. Listerolysin O from *Listeria monocytogenes*

appears to attack the membrane of the phagosome but not the cell membrane permitting the bacteria to proliferate in the cytosol without killing the cell (Schnupf and Portnoy, 2007). It also promotes *L. monocytogenes* replication in the phagosome (Birmingham et al., 2008). Alpha-hemolysin and Lysterolysin O also hi-jack host factors for bacterial benefit (Inoshima et al., 2011; Ribet et al., 2010). It remains to be seen whether NetB fulfils these types of roles to promote *C. perfringens* colonisation of the broiler intestine. *In vitro* experiments with bone marrow derived macrophages and dendritic cells could provide insight into whether *C. perfringens* are phagocytosed and undergo degradation and then antigen presentation. The use of isolates such as the CP1 and CP1ΔnetB mutant in these experiments could determine a role for NetB. Cytokine production, bacterial survival, cell chemotaxis as well as host cell survival could also be investigated to elucidate the functions of this key virulence factor.

Little is known about the pore-forming toxin, NetB. Currently, it is not yet known which component of the cell membrane NetB binds with although it is more active when there is a source of cholesterol (Savva et al., 2013). NetB positive culture supernatants are reported to be cytotoxic to the avian LMH cell line but not to Vero cells (African green monkey kidney cell line), DF-1 cells (chicken embryo fibroblast cell line) or HD-11 cells (chicken macrophage-like cells)(Keyburn et al., 2008). This work indicates that NetB has an affinity for avian epithelial-like cells but further study is required with primary cell cultures to fully determine this. Defining a receptor for this toxin could explain whether NetB targets a specific cell type or has varying roles

depending on the cell type it comes in contact with. *C. perfringens* β-toxin was recently shown to interact with the $P2X_7$ receptor on THP-1 cells, a human monocytic cell line. Antagonists of this receptor block cytotoxicity induced by β-toxin *in vitro* and also prevented death in an *in vivo* mouse model (Nagahama et al., 2015b). Determining the favoured receptor for NetB on avian cells could allow for the discovery of inhibitory molecules which prevent cell death and inflammation. These could then be used as treatments or preventative measures for NE.

6.6. Breed differences to *C. perfringens*

The experiments carried out as part of this thesis have aimed to inform the immune cell response to *C. perfringens* antigens in three commercial broiler breeds. Chapters 2, 3 and 5 used the Ross 308 broiler whereas Chapter 4 used Cobb and Hubbard broilers. In all experiments the presence of heterophils was quantified. Heterophils are present in microscopic NE lesions as well as mononuclear cells (Cooper et al., 2013; Kaldhusdal et al., 1995). Increased numbers of heterophils were detected in loops containing bacteria in Chapter 3 but this was not the case in Chapter 4 where there was no change in comparison to the control. In both cases isolates which differ in their virulence were used. It may be possible that the Ross birds used in Chapter 3 had an increased heterophil response to the presence of *C. perfringens* bacterial cells in comparison to Cobb and Hubbard birds but as the same isolates of bacteria were not used in both experiments these cannot be directly compared.

We investigated the population of other cells which respond early in infections, KUL01+ cells and γδ T cells. The aim of this was to determine whether the cellular response was similar across two commercial breeds. The most striking difference, however, was the difference between the two commercial breeds used in Chapter 4, with Hubbard broilers having more γδ T cells and macrophages/dendritic cells than their Cobb counterparts. To our knowledge there have been no other studies quantifying the immune cell types in the duodenum after *C. perfringens* challenge. There have, however, been investigations into immune cell infiltration to the intestine during *Eimeria ssp*. challenge using immunohistology and flow cytometry (Choi and Lillehoj, 2000; Laurent et al., 2001; Lillehoj and Trout, 1996; Rothwell et al., 1995; Vervelde et al., 1996). Notably, γδ T cells increase soon after infection in the epithelial layer and the lamina propria (Choi and Lillehoj, 2000; Rothwell et al., 1995). Our work showed that Hubbard broilers had a significantly larger area positive for this cell type than Cobb broilers. It is possible that this increased immune cell population could contribute to protection against co-infection with *Eimeria* and *C. perfringens* in the Hubbard broiler. The reduced weight gain during NE is not as pronounced in Hubbard broilers and the antibody response is reduced in comparison to Cobb birds during co-infection challenge.

As many NE infection models include co-infection with *E. maxima*, *E acervulina* or an overdose of Paracox vaccine it is unclear whether changes in the intestinal immune cell responses after *Eimeria* infection contribute to NE. Cells responding to *Eimeria* parasites may not be able to respond fully to

a bacterial infection at the same time. Future work investigating the changes in immune cell populations over time to *C. perfringens* challenge as well as in co-infection models could inform disease pathogenesis and highlight which cells are involved in protective immune responses. This could benefit vaccine strategies for NE in the future as adjuvants could be chosen to stimulate the immune response in a particular way.

In addition to the numbers of various cell types identified in the broilers, we also aimed to assess their activity. IL-17A, IL-17F and IFN-γ mRNA expression was measured as some of the initial pro-inflammatory cytokines produced from γδ T cells and IL-6, IL-1β, CXCLi1 and CXCLi2 mRNA as cytokines from macrophages. Despite Hubbard broilers having higher populations of macrophages and γδ T cells there was no change in the cytokine mRNA between the two breeds measured in Chapter 4. It was expected that as more cells were present in Hubbard birds that they would also have higher cytokine responses.

It may be possible that cells present in Cobb broilers are more active than those present in the Hubbard. The genetics of the broilers may play a role in immune cell activity. Genetic susceptibility to NE has been previously mentioned. Cobb broilers had impaired productivity as well as increased lesion scores and antibody titres in comparison to Hubbard and Ross broilers following infection with *Eimeria* and *C. perfringens* (Jang et al., 2013). As well as this work with commercial broilers, in-bred lines of chicken have also been used in co-challenge NE models. These birds originated from the Avian Disease and Oncology Laboratory (ADOL) laboratory in the USA and have

previously been characterised to be resistant or susceptible to a number of diseases. For example, Line 6 is Marek's disease virus resistant and Line 7 is susceptible for the same virus (Bacon et al., 2000). Line 6 and 7 both have genetically identical MHC molecules, B_2, indicating that the difference is not dependant on the antigen presentation complex in these two lines. These birds do have different immunoglobulin G allotypes which could induce differences in adaptive antibody responses. In terms of NE, Line 6 is reported to have a reduced weight gain when co-challenged with *E. maxima* and *C. perfringens* in comparison to Line 7 (D. K. Kim et al., 2014). Despite having the same MHC molecules there could still be variation between these lines in the expression of innate barrier molecules, cytokine and chemokine response, antigen up-take and adaptive responses to the antigens presented on the MHC molecules but these birds could provide a useful tool in the investigation of genetic susceptibility.

It has yet to be seen whether an improved innate response could in fact defend against NE but it has been effective against other pathogens, including Salmonella (Swaggerty et al., 2014, 2009, 2008, 2004). Two broiler breeder lines, which differ in their innate immune response, have altered responses to *E. tenella* (Swaggerty et al., 2011). Breed A, characterised in previous studies to have a more robust innate response (Ferro et al., 2004; Swaggerty et al., 2004), had reduced *Eimeria* intestinal lesion scores accompanied with increased weight gain compared with Breed B. Resistance against *Eimeria* parasites is also likely to benefit resistance to NE since *Eimeria* is considered a pre-disposing factor for NE (Bangoura et al., 2014).

The breeds used by Swaggerty et al., (2011) were not named directly for confidentiality making it difficult to compare directly with our work. Jang et al., (2013) did not score *Eimeria* lesions but Hubbard broiler body weight gain was not affected to the same extent of that in Cobb broilers indicating the Hubbards may be more like Breed A and the Cobb like Breed B. Work with commercial breeds and in-bred bird lines does indicate a role for genetic resistance to NE and this could be a future avenue for study.

6.7. Mucosal barrier molecules in the presence of *C. perfringens* antigens

Barrier molecules, such as mucins are the first levels of protection in the broiler intestine; as a consequence a number of studies have investigated their activity post *C. perfringens* challenge, which is variable between studies (Calefi et al., 2014; Collier et al., 2008; Forder et al., 2012; Golder et al., 2011; Kitessa et al., 2014; Liu et al., 2012). These differences may depend on the type of challenge model used and other environmental factors such as host diet. Here, we detected differences across the two loops experiments where mucin mRNA was measured. Virulent and avirulent *C. perfringens* increased Muc2 and Muc5AC mRNA expression (Chapter 3) whereas the presence of bacteria had no effect on Muc5AC in Chapter 4. The presence of NetB, however, reduced Muc2 and Muc13. The role of mucins in NE pathogenesis is unclear as increased levels of Muc2 after *E. acervulina* and *E. maxima* infection appears to promote *C. perfringens* growth (Collier et al., 2008). It remains to be understood whether *C. perfringens* can use any of these molecules to its advantage during proliferation. Of the transmembrane

mucins found in the chicken genome, Muc13 is the only one which has been previously investigated in relation to NE. The chicken has fewer transmembrane mucin genes than humans or mice with only four being identified (Lang et al., 2006). One hypothesis could be that NetB+ *C. perfringens* actively reduces the expression of transmembrane mucins providing easier access to the enterocyte cell membrane for invasion. As these molecules are alternatively expressed for a number of reasons, including variations in diet and co-infections, *in vitro* methods, such as newly developed 3D culture systems, may provide novel methods of investigating the role of mucins after exposure to NetB.

2D monolayer cell culture would provide some initial answers but a 3D system could be a better alternative (Figure 6.3). Caco-2 cells, which are derived from human colon carcinoma cells, cultured on a collagen scaffold of the human villi structure showed increased levels of the transmembrane mucin, Muc17. Cells in this 3D system also had improved cell-to-cell tight junctions providing a stronger barrier against bacterial challenge compared with a monolayer culture of the same cell type. Cells cultured on the scaffold were resistant to bacterial challenge when monolayer cells were not (Kim et al., 2014). This method would require a chicken intestinal epithelial cell line to be utilised for the purposes of investigating host responses.

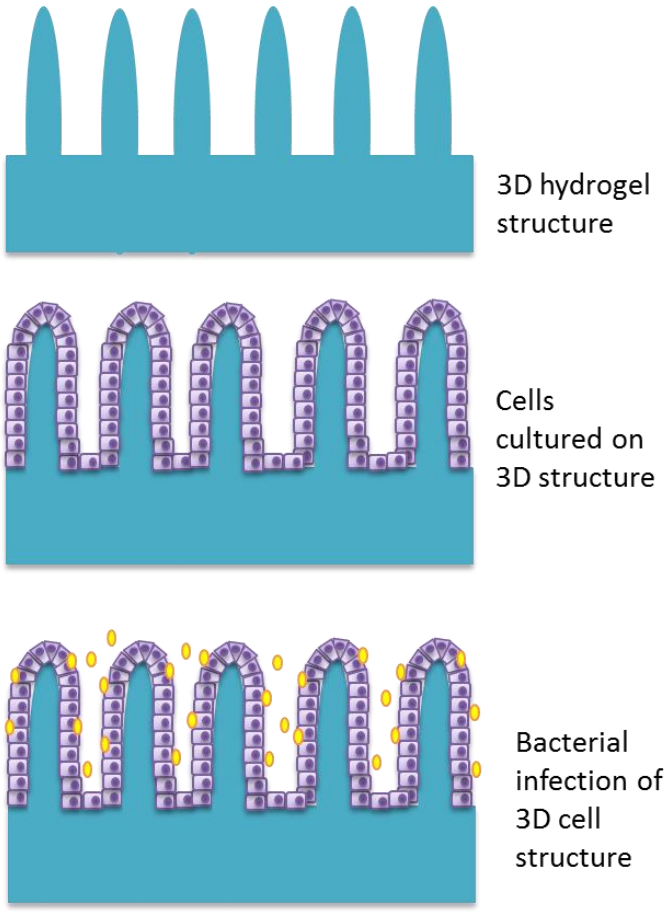

Figure 6.3. Schematic diagram of 3D scaffold culture system

The 3D hydrogel structure is prepared with collagen from a model of a villus structure. The intestinal cell line is cultured on this structure and can then be used for experiments such as bacterial invasion. Figure adapted from Kim et al., 2014.

The development of intestinal organoid cultures may also provide a greater insight into the response of mucin molecules and pro-inflammatory mediators (Bermudez-Brito et al., 2013; Leushacke and Barker, 2014). Intestinal organoid cultures are derived from pluripotent stem cells. When given the correct growth factors these stem cells develop into 3D structures

consisting of crypt like zones and columnar epithelial villus regions which have enterocytes, goblet cells and enteroendocrine cells (Figure 6.4). Human intestinal organoids have begun to be used for investigating host-pathogen interactions. *Salmonella enterica* was able to invade organoid epithelial cells and induce pro-inflammatory cytokine expression at the transcription and protein level (Forbester et al., 2015). *C. difficile* numbers reduced in the intestinal organoids after injection of a vegetative culture but some did remain viable for 12h. *C. difficile* and Toxin A, from this bacterium, injected in to the intestinal organoid disrupted the epithelium and therefore barrier function was lost (Leslie et al., 2015).

Similar experiments could be carried out with chicken intestinal organoids to investigate the effect of *C. perfringens* on the epithelial layer. These could also be used to determine whether the presence of NetB alters the expression of mucin molecules as well as molecules related to other functions such as the inflammatory response and cell viability. If mucins were found to be important in the defence against NE it may be possible to manipulate their expression to benefit the broiler via the use of probiotics (Mack et al., 1999; Plaza-Diaz, 2014; Smirnov et al., 2005) and management practices (Smirnov et al., 2004).

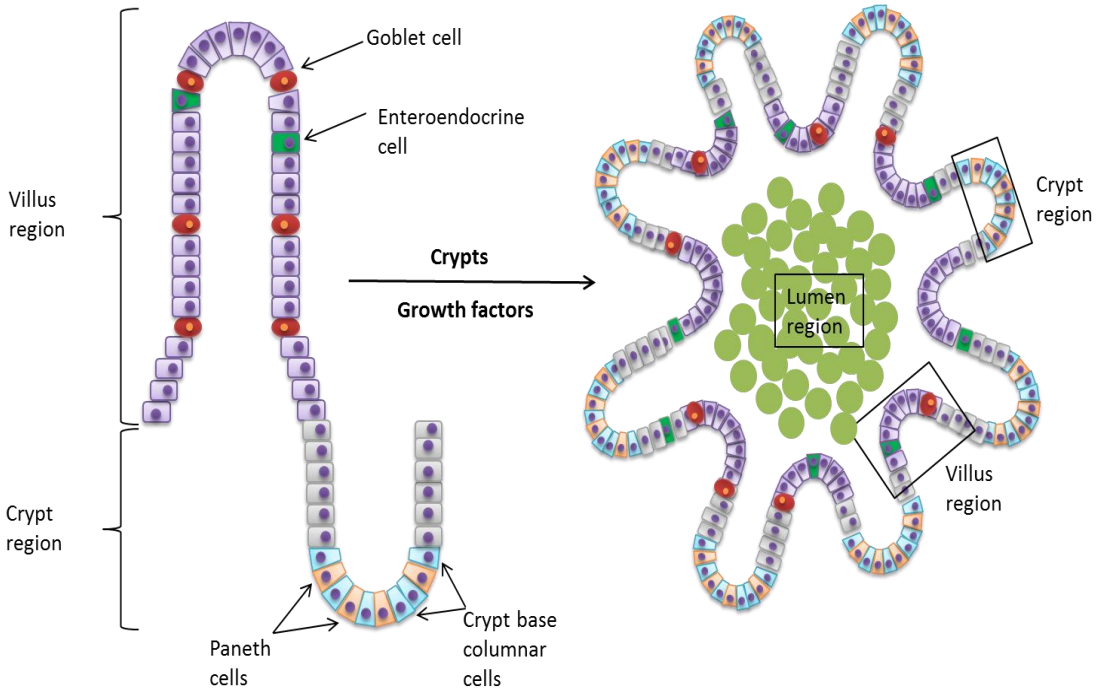

Figure 6.4. Schematic diagram of intestinal organoid

Intestinal organoids can be generated from a portion of a crypt or single pluripotent stem cells. Differentiated cells are found in the villus domain while stem cells (blue cells) and Paneth cells (orange cells) are found in the crypt domain of the organoid. Cells move up the crypt, differentiating as they reach the villus domain, and are shed into the lumen. Bacterial cultures can be injected into the lumen region. Figure adapted from Leushacke and Barker, 2014.

6.8. Cell Death in the presence of *C. perfringens* antigens

3D culture systems, like intestinal organoids, could also be utilised to investigate cell death in the presence of *C. perfringens*. Cell death via necrosis (un-regulated cell death) is considered to play a large part in NE as the many toxins produced by *C. perfringens* damage cells they come in contact with (Olkowski et al., 2008). In our experiments we investigated FAS and GIMAP8 mRNA expression; which are two genes related to cell death. FAS is associated with pro-apoptosis whereas GIMAP8 has been thought to be anti-apoptotic (Krücken et al., 2005; Lavrik and Krammer, 2012). The expression of these two genes varied across the loop experiments. There was no difference in GIMAP8 mRNA expression after the infusion of culture supernatant (Chapter 2) but expression was reduced in the presence of bacteria (Chapter 3). FAS mRNA expression was increased in the presence of CP4 and CP5 isolates but not culture supernatant from these isolates (Chapter 3). On the contrary, FAS mRNA expression was reduced in the presence of CP1 wild type (NetB+) and CP1ΔnetB (NetB-). These results contradict each other and there are many other factors of the cell death pathways involved which may have provided further answers. Some of the possible cell death mechanisms are apoptosis, necrosis and, an intermediate of the two, necroptosis. Binding of FAS with its ligand initiates the death receptor complex (Lavrik and Krammer, 2012). This in turn activates molecules from the Caspase cascade which are predominantly associated with apoptosis. Measuring downstream Caspase activity could have indicated apoptotic cell death. When Caspase is inhibited, necroptosis is activated via the molecules RIP1 and/or RIP3 and further investigation of these could have

indicated this mechanism (Gunther et al., 2013). Necrosis is considered generally to be uncontrolled and results in the sudden loss of membrane integrity causing the release of cellular contents and inflammation inducing danger associated molecular patterns (DAMPs) (Davidovich et al., 2014).

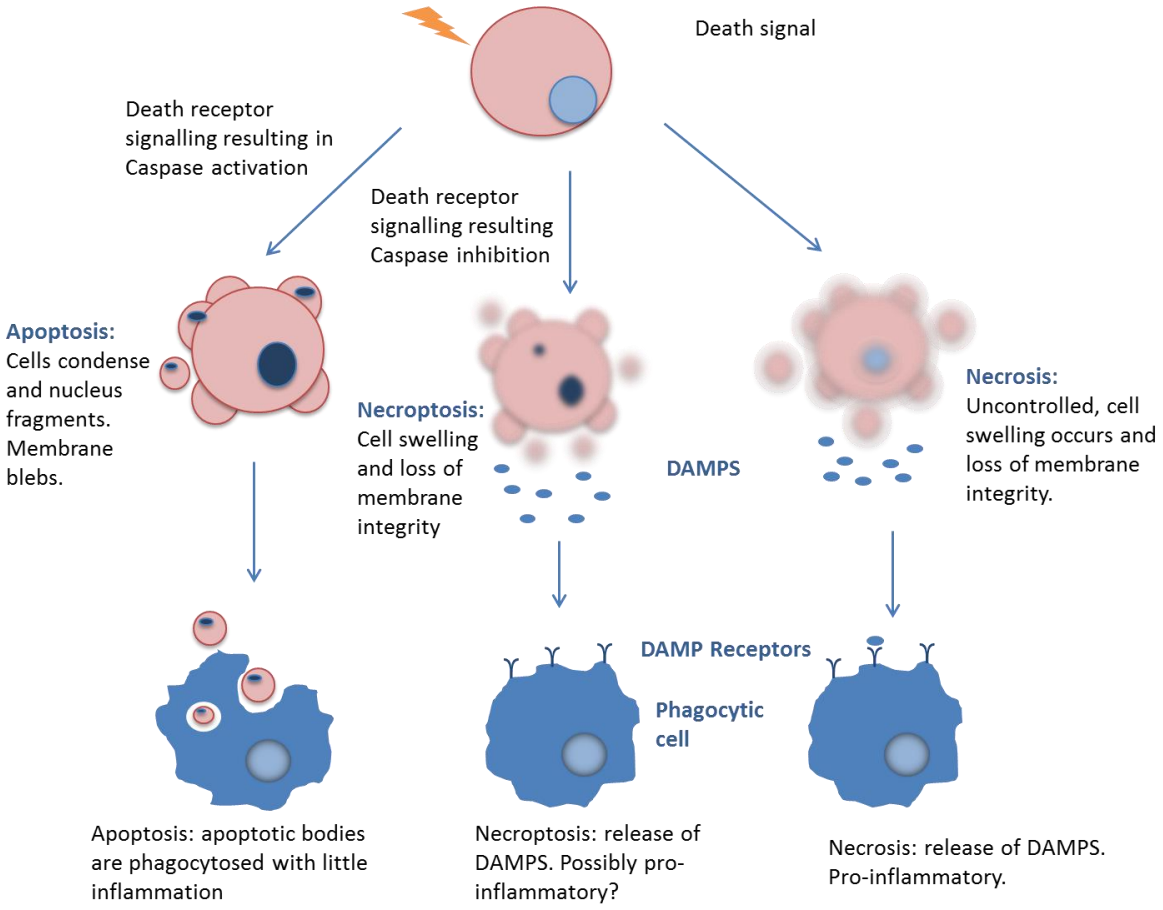

Figure 6.5. Cell death mechanisms

Cell death mechanisms possibly activated during *C. perfringens* toxin exposure. Adapted from Davidovich *et al.* (2014).

Increased FAS and reduced GIMAP8 mRNA expression (Chapter 3) indicated apoptotic or necroptotic cell death as both can be initiated via this death receptor. Apoptosis was detected in the duodenum, jejunum and ileum in *C. perfringens* challenged broilers via the TUNEL method which detects

fragments of DNA in tissue sections (Liu et al., 2012). BCL2 mRNA expression in the spleen and mucosal layer of the susceptible 6.3 inbred line was increased compared with the 7.2 resistant line after an *E. maxima* and *C. perfringens* NE challenge (Dinh et al., 2014). BCL2 is anti-apoptotic therefore, increased levels of this gene are associated with cell survival (Adams, 1998). Promoting cell survival, in this case, does not appear to be beneficial for NE challeged birds. Apoptosis is considered to release fewer DAMPs than necroptosis or necrosis (Kaczmarek et al., 2013) so it may be possible that in Line 6.3 the correct inflammatory mediators are not produced to initiate a protective immune response. In Chapter 4 we did not detect any difference in FAS mRNA expression between the two commercial breeds used but further investigation of cell death mechanisms during NE challenge may interesting to investigate in the two inbred lines.

As we do not detect consistent results between studies it is possible that various cell death mechanisms are involved in NE. Further investigation may provide knowledge on the role of pore-forming toxins in the chicken and the mechanisms by which cells die. It has been noted, recently, that pore forming toxins may initiate necroptosis (Essmann et al., 2003; Kennedy et al., 2009; Knapp et al., 2010). β-toxin, from *C. perfringens* Type B, activates this version of programmed cell death. Porcine endothelial cells incubated with β-toxin showed microscopic effects of necrosis, such as chromatin condensation, swelling of intracellular organelles and plasma membrane disruption. Incubation of these cells with an inhibitor of necroptosis, Necrostatin-1, prevented cell death in the presence of β-toxin. As NetB has

some structural similarities to *C. perfringens* β-toxin it may be possible that this NE virulence factor causes necroptosis rather than/as well as necrosis in the intestine of infected broilers. Increased investigation into cell death during NE could provide a greater understanding of disease pathogenesis, bacterial evasion strategies and host responses to induce inflammation and protection during infection which could in turn lead to further advances in treatment or disease prevention.

6.9. NE Disease Challenge Models

Much of the experimental work in this thesis used the duodenal ligated loop model to detect differences in the early responses to *C. perfringens* and its virulence factors. A similar model using ligated loops in the duodenum of broilers has also been used to investigate iron bio-availability (Tako et al., 2010). Despite being a useful tool for investigating host-pathogen interactions this method does have limitations. In each of the ligated loop experiments in this thesis there were histological changes in the control loops. The surgical procedure around the duodenal loop, avoiding the pancreas does induce some damage to the intestine. Increasing the number of loops to six in Chapter 3 did result in increased damage overall and this should be taken into account when designing other studies in future. Other areas of the intestine may incur less damage during the surgical procedure, such as the jejunum. This may also allow for spacer loops which are used in ligated loop models in other species (Girard-Misguich et al., 2011; Maluta et al., 2014). Although confident that there was no transfer of material between

loops in out studies these spacer loops would provide an extra barrier against contamination across loops.

Investigating host responses requires robust experimental challenge models where the consequences of the disease and the impact of treatments can be noticeably detected. Various infection models have been described in the literature and were reviewed by Shojadoost et al., (2012). Most of the previous attempts to experimentally reproduce NE at our facility have failed, with the exception of a co-infection model with *Eimeria,* infectious bursal disease virus and *C. perfringens* (Saleem, 2013) therefore, the final aim of this thesis was to establish a protocol for inducing NE by administering multiple doses of *C. perfringens* in conjunction with an increase in dietary protein used by collaborators (Chapter 5). Unfortunately we were unable to consistently reproduce NE with the protocols used. It is possible that this was in part due environmental factors such as stocking density. Recent studies have investigated the role of dietary fishmeal and co-challenge with *Eimeria ssp*. on the development of NE and how these pre-disposing factors impact on the cecal microbiota (Stanley et al., 2014; Wu et al., 2014). The ceca samples microbes from both descending and ascending gastrointestinal contents via regular peristalsis and retrograde intestinal movements (Sklan et al., 1978; Stanley et al., 2015). The ceca are the main site of colonisation for *C. perfringens* in healthy birds. It is therefore probable that any effect pre-disposing factors have on the gastrointestinal microbiota would be detected in the ceca. Changes in the cecal microbiota have been detected after the inclusion of both fishmeal and *Eimeria* when used as pre-disposing factors

(Wu et al., 2014). Although, a single dose of *C. perfringens* did not appear to alter the cecal microbiota. Changes were only detected after *C. perfringens* challenge in the presence of fishmeal and *Eimeria* infection (Stanley et al., 2014).

Rodgers et al., 2015 carried out multifactorial analysis on a challenge model carried out in 1344 Ross broiler birds administered fishmeal*, Eimeria* challenge *(acervulina, and maxima)*, and *C. perfringens* to determine the effect each has on the induction of NE. *Eimeria* challenge does significantly increase the lesion score of broilers whereas the inclusion of fishmeal did not impact the lesion score. Birds given *C. perfringens* without other predisposing factors had low lesion scores post mortem.This is not unexpected as there were two doses of *C. perfringens*; in general, studies where *C. perfringens* alone was used to induce NE, multiple doses of bacterial culture are offered over a number of days (3-7). The lack of changes in the cecal microbiota after *C. perfringens* administration may also be in part down to the single dose used.

These studies begin to evaluate the factors required for *C. perfringens* to proliferate in the intestine of chickens. It is likely that technologies, such as next generation sequencing, used in these studies may provide a wider insight to the changes that occur in the host in the lead up to NE development. It may be interesting to also investigate the microbiota composition under a number of other stressors (e.g. heat stress or high stocking density) in relation to NE. This may highlight whether immune and environmental stressors all induce the same alterations in microbiota that

lead to NE or whether *C. perfringens* is opportunistic in a number of scenarios.

6.10. Conclusions

Throughout this thesis we have detected a number of changes in host responses to *C. perfringens* and the toxins it produces. Using the *in situ* intestinal loop model we have identified that *C. perfringens* may have some host evasion mechanisms and that these could be assisted by the NetB toxin. As identified earlier in this chapter, the role of NetB in NE is still to be fully understood. Determining how this toxin interacts with antigen recognition receptors and innate cells in the intestine would improve knowledge on disease pathogenesis.

To date, the presence NetB in suspected cases of NE is not used as part of the criteria for diagnosis of birds in veterinary laboratories but diagnosis is based on a number of criteria. To diagnose NE, lesions should be detected macro- and microscopically, *C. perfringens* should be isolated from the intestinal tract and alpha-toxin confirmed in the intestinal contents. Only if these criteria are met should NE be diagnosed (Cooper et al., 2013). Unfortunately, these tests can only be carried out after mortalities have occurred in a flock and do not take into account the presence of NetB. A test for the presence of *C. perfringens* and NetB in faecal samples of suspected subclinical NE flocks could be beneficial to the broiler industry. The ability to utilise faecal samples would make the test non-invasive and the presence of NetB toxin would more reliably indicate the presence of pathogenic *C. perfringens* isolates and therefore NE associated disease.

It may be possible to use impedance-based technology for diagnosis as an assay detecting *C. difficile* toxin in clinical stool samples has been developed (Huang et al., 2014; Ryder et al., 2010). In the modified version of the original assay for Toxin B from *C. difficile*, the stool samples are enriched using an immunomagnetic separation which removes other non-target proteins. This assay is non-invasive, provides real-time analysis and quantification of the levels of toxin present in the stool samples. The main drawback of this assay was the time required to carry out this assay which was around 60 hours (Huang et al., 2014). Another RTCA assay has been developed for the detection of *Vibrio cholera* toxin. In this assay, the toxin was detected within two hours (Jin et al., 2013). A test such as this could provide some useful information during instances of reduced performance in the field. For example, flocks with reduced performance and high levels of NetB detected in the faeces could be treated with an antimicrobial targeted for *C. perfringens*. Initial steps for the development of a NetB assay would be to confirm the presence of this toxin in the faeces of experimentally challenged broilers.

There are still many avenues of study for NE in broilers. As *in vitro* tools, such as monoclonal antibodies for cytokines, are developed for investigating the chicken immune response it will become possible to investigate inflammatory mediators at the protein level as well as at the transcription level. This may also make it easier to identify which cells are producing these mediators.

We have shown that different broiler breeds have different immune cell populations in their intestine. In relation to *C. perfringens* there was no difference in the innate inflammatory mediators measured as part of this study. A wider experiment is required to investigate the protective responses shown by the different breeds. It may be important to compare cell responses over time after *C. perfringens* challenge and a co-infection model with *Eimeria ssp.* and *C. perfringens*. This could help to target vaccines for a particular arm of the adaptive immune response.

Our experiment using culture supernatant with and without *C. perfringens* cells showed a greater inflammatory response in the presence of bacteria. One possible strategy for NE vaccination could be to combine both membrane and toxin proteins by the use of membrane vesicles (MVs). These vesicles bud off from bacterial cells and contain various factors, such as LPS, lipoproteins, and proteins from the cytoplasm (Lee et al., 2009). These structures were once considered by-products of bacterial culture but recent findings have indicated that these structures are released by bacteria and can manipulate the host immune response (Kaparakis-Liaskos and Ferrero, 2015). It may be possible to develop vaccines using MVs for infectious disease as they contain immunogenic proteins and have their own adjuvant activity (Kaparakis-Liaskos and Ferrero, 2015). MVs are produced by *C. perfringens* and can contain a number of different proteins as well as DNA and RNA (Gurung et al., 2011; Jiang et al., 2014). MVs can also be engineered to contain specific proteins important for immunity (Baker et al., 2014; Fantappiè et al., 2014). It may be possible to develop MVs containing

proteins that will allow birds to develop antibodies against NetB and other *C. perfringens* toxins which could then be used as a vaccine candidate. Further screening of immunogenic protective antigens may be required to ensure good candidate proteins for MVs.

Some of our mRNA expression data as well as that of other studies have indicated various cell death mechanisms play a role in NE disease pathogenesis. Currently, it is not clear which mechanism is mainly involved and whether this is induced by the bacteria and the toxins it produces or the host to influence protection and instigate other responses. This could be investigated using *in vitro* experiments with different cell types and 3D culture systems. Single toxin preparations (e.g. NetB) as well as combinations (e.g. NetB and TpeL), preparations with *C. perfringens* cells or membrane vesicles could be used to compare differences in cytotoxicity.

We have shown changes soon after exposure to *C. perfringens* but it is still unclear how these relate to experimental challenge models of NE. Full characterisation of the immune response during NE challenge would greatly benefit vaccine and treatment development in the future.

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